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OBITUARY



Ferenc Holló (1923–1997)

Dr **Ferenc Holló,** Titular Professor, Editor-in-Chief of *Magyar Állatorvosok Lapja* (Hungarian Veterinary Journal) died on 4 December 1997 of a sudden heart attack at the age of 74. His death is a grievous loss to the entire Hungarian veterinary profession, as with him the leading personality and an indefatigable worker of Hungarian veterinary journalism has departed this life, an outstanding journalist and editor who did his work with great devotion and expertise over a period of almost five decades.

Ferenc Holló was born in Jászberény on 26 August 1923. He survived the Second World War as a prisoner at the Mauthausen concentration camp. He took his veterinarian's diploma in Budapest in 1947. In the initial phase of his career, Ferenc Holló specialised in parasitology and spent eight years working at the Central Veterinary Institute as a laboratory parasitologist. In 1960 he became a research fellow at the Research Laboratory of Helminthology, established within the Department of Parasitology of the Budapest University of Veterinary Science. From 1980, he spent two and a half years in Algeria as a parasitology expert of the UNDP/FAO, and organised the first veterinary parasitological diagnostic laboratory of the country. At the Budapest University of Veterinary Science he became the lecturer of the optional subject "Tropical Parasitoses", and was appointed titular professor.

MAGYAR TUDOMÁNYOS AKADÉMIA KÖNYVTÁRA

As a research parasitologist, he was mainly concerned with practical aspects of the control of helminthoses, such as diagnosis, therapy, and systematic prevention. He was awarded the degree Candidate of Veterinary Science (Ph.D.) in 1965 for his thesis written about metastrongylosis of pigs. His most important work was the book entitled "Veterinary Parasitological Diagnostics", which he co-authored with Dr László Nemeséri. It ran into three Hungarian-language editions (1957, 1961, 1972) and was published in German (1964) and Spanish (1966) language as well. Ferenc Holló co-edited (together with Csaba Gallyas) the "Veterinary Explanatory Dictionary", published in Hungarian language in 1984.

Dr Holló joined in the work of editing the monthly periodical *Magyar Állatorvosok Lapja* (Hungarian Veterinary Journal) in 1949. He learned the art of high-standard scientific journalism from Professor Sándor Kotlán, who was the editor of the journal at that time. Subsequently, editing gradually came into prominence within Ferenc Holló's curriculum: up to 1961 he acted as editor and from that time until his death as editor-in-chief of the journal. His work as editor coincided with the hard period of profound economic and political changes affecting Hungarian agriculture and animal breeding. With his deep professional commitment, refined flair for diplomacy, and personal style facilitating the resolution of conflicts he managed to shape the journal so that it always addressed timely problems of the veterinary profession and professional policy. He belonged to the type of "active editor": in addition to using the manuscripts submitted to the journal, he tended to anticipate important topics of the future and often invited papers from specialists so that his periodical could reflect the progress of science and efficiently serve its most important purpose, i.e. postgraduate veterinary education and providing assistance to veterinary practitioners in performing their tasks. Owing to his high-standard editorial work, the articles of this monthly periodical published in Hungarian language, with abstracts in foreign languages, have been followed with attention and cited by many people both within and outside Hungary.

Dr Holló actively participated in the scientific public life. He was an industrious member of the Presidium of the Hungarian Veterinary Society as well as of the Hungarian Society of Parasitologists. He was decorated with the Bronze Medal (1968) and Silver Medal (1978) of the Order of Work, the "Ferenc Hutyra" Memorial Medal (1976), the Commemorative Medal of the Hungarian Society of Parasitologists (1977), and the "Sándor Tolnay" Prize (1994).

As an enthusiastic supporter of the international co-operation between the editors of veterinary journals, he played an active role in the European Association of Veterinary Editors, and cultivated friendly bilateral relations with the editors of sister journals of the neighbouring countries as well as with those of German and French veterinary journals.

He was a just person who had a high opinion of friendship and team-spirit. He liked to discover values and positive properties in others and was happy if he could show his appreciation for them. We will miss not only his precise and reliable editorial work but also his kind, genial and warm humanity. Ferenc Holló has made his mark in the history of Hungarian veterinary technical journalism for ever.

December 1997

Tibor KASSAI

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EFFECT OF IMMUNOSTIMULATION ON CYTOTOXIC ACTIVITY OF INTESTINAL INTRAEPITHELIAL LYMPHOCYTES OF CHICKENS IN INFECTIOUS BURSAL DISEASE AND *EIMERIA TENELLA* INFECTIONS

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In chickens, intestinal intraepithelial lymphocytes (iIELs) exhibit spontaneous natural killer (NK) cell like activity, by which they are active in the first line of defence on gut epithelial linings. In the present study, the cytotoxic activity of iIELs was found to be drastically suppressed in chickens experimentally infected with infectious bursal disease (IBD) virus at the age of 5 weeks and also in chickens experimentally infected with *Eimeria tenella* oocysts at the age of 8 weeks ($p < 0.01$). As nonspecific immunostimulation is gaining importance, immunostimulants such as immunostimulating *Mycobacterium phlei* (ISMP) and bone marrow culture supernatant (BMCS) were tested for their influence on the functional activity of iIELs of chickens in IBD and *E. tenella* infections. In chickens primed with ISMP a week prior to respective experimental infections, it was found that the cytotoxic activity of iIELs was restored ($p < 0.01$) in both IBD and *E. tenella* infections. At the same time, in chickens primed with BMCS a week prior to respective experimental infections, the cytotoxic activity of iIELs was restored to a certain extent ($p < 0.01$) in *E. tenella* but not at all in IBD infection. These results showed that application of immunostimulation helped potentiate and restore the functional activity of iIELs of chickens in IBD and *E. tenella* infections.

Key words: *Eimeria tenella*, infectious bursal disease (IBD), intestinal intraepithelial lymphocytes (iIELs), immunostimulation, natural killer (NK) cells

In chickens, intestinal intraepithelial lymphocytes (iIELs) constitute an important cellular component of gut-associated lymphoid tissue (GALT). The majority of them are Υδ T lymphocytes that exhibit NK cell like cytotoxic activity (Chai and Lillehoj, 1988), which is spontaneous, not MHC restricted and without being primed. This functional activity of chicken iIELs was earlier described in rotavirus (Myers and Schat, 1990) and eimerian (Lillehoj, 1989) infections. A number of studies conducted in mice explored different possible ways of recognising and elimi-

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nating foreign antigens by iIELs and also their cytokine secretory profile that aids in this process (Klein and Hamad, 1995).

Potentiating the host immune status by various nonspecifically acting biological, chemical and pharmacological immunostimulants is gaining importance (Hadden, 1993). Hence, a wide variety of bacterial and immunopharmacologically active compounds have been tested for their activity in livestock (Blecha, 1988). Immunostimulating *Mycobacterium phlei* (ISMP), a bacterial lysate has been found to act as a good nonspecific immunostimulant in several animal species in different infections (Mallick et al., 1985; Tewari et al., 1996). Similarly BMCS, a crude mixture of cytokines obtained in bone marrow cultures, has also been found to act as a good immunostimulant in rodents (Ghosal et al., 1990) and chicks (unpublished observation, Ghosal, 1994).

Immunosuppression in chickens infected with IBD is well documented, predisposing the infected birds to a number of secondary pathogens (Hirai et al., 1974). In avian coccidiosis caused by *Eimeria* spp., the role of iIELs has been identified to be associated with coccidial biology by *in situ* transportation of sporozoite stages besides their role as effector cells in anticoccidial immunity (Lawn and Rose, 1982; Lillehoj, 1989; Lillehoj and Bacon, 1991). The present study evaluates the cytotoxic activity of chicken iIELs in experimental IBD and *Eimeria tenella* infections and also the immunostimulating activity of ISMP and BMCS on the cytotoxic activity of iIELs of chicken in these infections.

Materials and methods

Experimental birds

Healthy, unvaccinated, one-day-old White Leghorn chicks procured from the Central Avian Research Institute (CARI), Izatnagar, were reared under hygienic conditions and provided with feed and water *ad libitum*. These chicks were reared in two groups. The first group consisted of 40 chicks. They were grown up to 5 weeks of age and used for studies on IBD. Twenty birds in this group were randomly picked up, killed, and bursal suspension was prepared from their bursa of Fabricius. The bursal homogenate prepared from each bird was tested against IBD hyperimmune sera by agar gel precipitation test (AGPT), so as to rule out any IBD infection in these birds. The findings of the AGPT revealed that these birds were free from IBD infection.

The second group consisted of 20 chicks. They were grown up to 8 weeks of age and used for studies on *E. tenella* infection. Faecal droppings of these birds were examined microscopically for any oocysts, so as to rule out any *E. tenella* infection. It was found that all these birds were free from *E. tenella* infection (Table 1).

Table 1

Experimental design

No. of birds	Group I				Group II			
	40*				20			
Subgroups	A	B	C	D	A	B	C	D
No. of birds	5	5	5	5	5	5	5	5

Group I

Group of chicks grown up to 5 weeks of age for studies on IBD.

* Twenty out of 40 chickens were randomly selected and tested for the presence of any IBD infection.

Subgroups

A: Normal, healthy chickens at the age of 5 weeks as controls.

B: Chickens experimentally infected with IBDV at the age of 5 weeks.

C: Chickens inoculated with ISMP one week prior to experimental IBD infection as in 'B'.

D: Chickens inoculated with BMCS one week prior to experimental IBD infection as in 'B'.

All these birds were killed 48 h after experimental IBD infection to study the functional activity of iIELs.

Group II

Group of chicks grown up to 8 weeks of age for studies on *E. tenella* and screened for the presence of any *E. tenella* infection

Subgroups

A: Normal, healthy chickens at the age of 8 weeks as controls

B: Chickens experimentally infected with *E. tenella* oocysts at the age of 8 weeks.

C: Chickens inoculated with ISMP one week prior to experimental *E. tenella* infection as in 'B'.

D: Chickens inoculated with BMCS one week prior to experimental *E. tenella* infection as in 'B'.

All these birds were killed 8 h after experimental *E. tenella* infection to study the functional activity of iIELs.

IBD virus

A field strain of IBD virus ($10^{4.5}$ $TCID_{50}$/ml) in the form of bursal homogenate procured from the Division of Avian Diseases, IVRI was used in 1:10 dilution.

E. tenella *oocysts*

A wild strain of *E. tenella* originally isolated from a field outbreak and established in pure line culture using single oocyst isolation protocol; supplied by the Division of Parasitology, IVRI was used.

Immunostimulants

Immunostimulating Mycobacterium phlei (ISMP). The ISMP was prepared by the method of Mallick et al. (1985). Briefly, *M. phlei* seed culture was inoculated and grown in Haffkin's flasks with glycerol nutrient broth. The lush bacterial growth was collected, dried at 37 °C, triturated and suspended in veronal buffer [with 0.15% trypsin (Sigma, USA)] at the rate of 80 mg/ml. Then, stirred on magnetic stirrer for 48 h and later passed through steam for one and half hour to inactivate residual trypsin. After the sterility and safety tests, its potential immunostimulating dose was standardised to 5 mg/chicken.

Bone marrow culture supernatant (BMCS). The BMCS was prepared by the methods of Petrov et al. (1980) and Ghosal et al. (1990) with slight modifications. Briefly, chicks of 3–4 weeks of age were inoculated with ISMP (5 mg/bird) and sacrificed after one week to prepare bone marrow cultures derived from femur explants. This culture was re-sensitised *in vitro* with ISMP (1.5 μg/ml of cell suspension) and incubated in culture bottles at 41 °C for 48 h under 5% CO_2 atmosphere in a CO_2 incubator. Protein content of the immunostimulating dose was standardised to 20 μg/chicken after safety tests in birds.

Agar gel precipitation test (AGPT)

The AGPT was done according to the method of Hirai et al. (1972) with slight modifications. The bursal homogenate suspensions prepared from the bursa of Fabricius of birds under test were tested against IBD hyperimmune sera to detect the presence/absence of any IBD viral antigen.

Experimental infection protocol

The IBD infection study was carried out in a group of chickens at the age of 5 weeks. The remaining 20 birds in the first group were subdivided into four subgroups of 5 birds each, of which one subgroup was kept as uninoculated,

healthy control. The IBD virus (1:10 dilution of $10^{4.5}$ $TCID_{50}$/ml of virus) in the form of bursal homogenate was inoculated orally at a rate of 0.2 ml/chicken to the other three subgroups. The birds inoculated with IBD virus were killed 48 h after experimental infection, their bodies were dissected and duodenal 'c' loops were processed for iIELs isolation. The establishment of experimental IBD infection was confirmed by AGPT using bursal homogenate of birds under test against IBD hyperimmune sera. The *E. tenella* infection study was carried out in a group of chickens at the age of 8 weeks. Twenty chickens in the second group were subdivided into 4 subgroups of 5 birds each, of which one subgroup was kept as uninoculated healthy control. The *E. tenella* oocysts were inoculated orally at a rate of 10^5 oocysts/chicken. The pathogenicity of *E. tenella* had been tested previously while passaging through chicks for pure line culture. At necropsy, haemorrhages were observed in the intestines of chicks used for passage and the mucosal scrapings from these birds were examined microscopically. *E. tenella* oocysts were observed in these mucosal samples. The chickens inoculated with *E. tenella* oocysts in the infection study were sacrificed 8 h post-infection, their bodies were dissected and duodenal 'c' loops were processed for the isolation of iIELs.

In both the IBD and the *E. tenella* infection studies, the immunostimulants ISMP (5 mg/bird intramuscularly) and BMCS (20 μg/bird intraperitoneally) were injected into birds of the respective subgroups a week prior to administering the respective infections, i.e. at 4 weeks in the IBD experiment and at 7 weeks in the *E. tenella* study (Table 1).

Isolation of iIELs

The iIELs were isolated from the chicken intestine by adopting the methods of Chai and Lillehoj (1988) and Hoggenmueller et al. (1993). In brief, duodenal 'c' loops were taken out from chickens after killing them by cervical dislocation, and processed for isolation of iIELs (Anand Kumar and Das, 1996). The total viable cell count of iIELs collected in the RPMI-1640 growth medium was expressed in millions/ml.

Cytotoxicity assays

Cytotoxicity assays were carried out by MTT colorimetric assay by adopting the original method of Espevik and Meyer (1986) with suitable modifications (Anand Kumar and Das, 1996). Vero cells were used as target cells and the iIELs isolated from the chickens were used as effector cells in these cytotoxicity assays.

Statistical analysis

Mean and standard error were used to calculate the mean percentage of cytotoxic activity of iIELs and a complete randomised design (CRD) was used to test the level of significance among different subgroups of experimental birds, in terms of the cytotoxic activity of iIELs.

Results

A marked depression in the functional activity of iIELs of chicken following experimental IBD infection was seen 48 h post-infection (significant at $p < 0.01$). The mean percentage cytotoxic activity of chicken iIELs was found to be 90.37 ± 2.53 and 23.62 ± 3.89 in healthy controls and IBD virus infected birds, respectively. Whereas, in chickens primed with ISMP, the cytotoxic activity of iIELs after 48 h of experimental IBD infection was found to be restored to 80.02 ± 9.39 (significant at $p < 0.01$). However, BMCS failed to restore the cytotoxic activity of iIELs in IBD infection. The cytotoxic activity of iIELs in chickens primed with BMCS was found to be 25.88 ± 3.81 after 48 h of experimental IBD infection (Fig. 1).

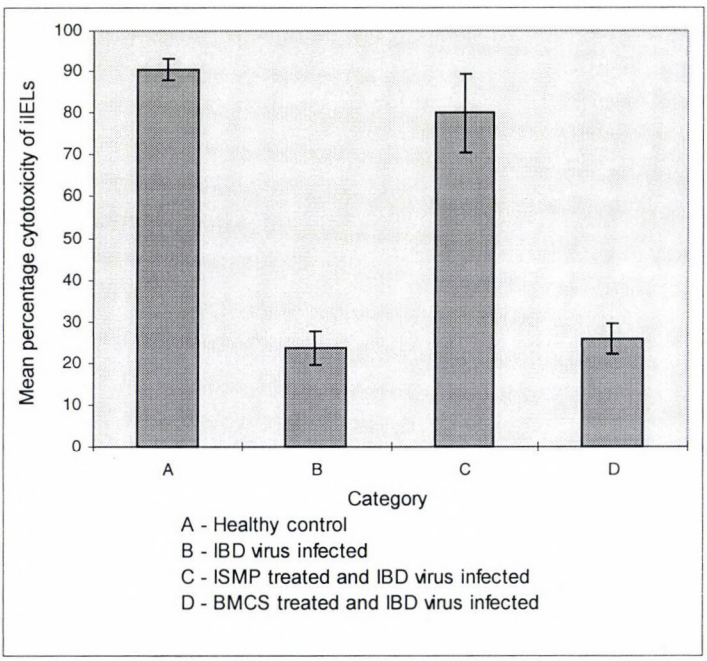

Fig. 1. Effect of immunomodulators on restoration of IBD virus induced depressed cytotoxic activity of chicken iIELs

A significant suppression in the functional activity of iIELs of chicken following *E. tenella* infection was seen 8 h after oocyst inoculation (significant at $p < 0.01$). The mean percentage of cytotoxic activity of iIELs of chicken was found to be 80.20 ± 3.45 and 27.96 ± 4.05 in healthy controls and *E. tenella* infected birds, respectively. However, this cytotoxic activity of iIELs was found to be restored (significant at $p < 0.01$) appreciably in chickens previously sensitised with either ISMP or BMCS and subsequently exposed to *E. tenella* infection with a mean percentage cytotoxic activity of iIELs as 64.52 ± 4.53 in ISMP treated and *E. tenella* infected birds and 55.36 ± 3.06 in BMCS treated and *E. tenella* infected birds (Fig. 2).

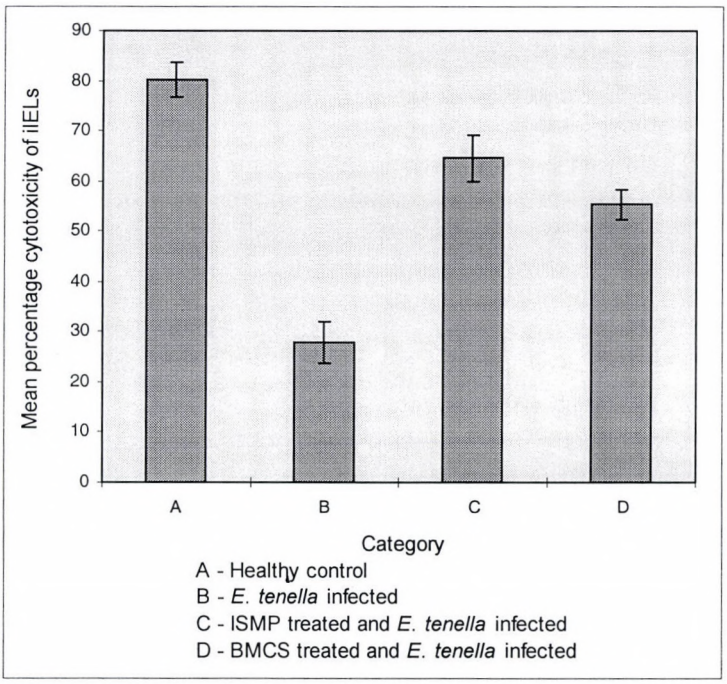

Fig. 2. Effect of immunomodulators on restoration of *E. tenella* induced depressed cytotoxic activity of chicken iIELs

Discussion

In chickens, iIELs of GALT exhibit NK cell like cytotoxic activity, by which they are active in the first line of defence in the gut epithelium (Ernst et al., 1985; Chai and Lillehoj, 1988). Their unique capacity of recognising and eliminating the foreign antigens and also their cytokine secretory profile con-

firms their role in local cell-mediated immunity (Klein, 1995). The iIELs are mainly Υδ T lymphocytes and to a certain extent non-T, non-B lymphocytes. However, a lineage of thymus-independent lymphocytes detected in the gut epithelium of chickens (Bucy et al., 1990), mice and humans (Klein, 1995) is attracting the attention of scientists studying the precise mechanism of foreign antigen recognition and elimination by iIELs.

IBD and coccidiosis are economically important diseases of poultry. Although humoral immune responses are chiefly affected in chickens infected with IBD (Allan et al., 1972; Faragher et al., 1974), there is some evidence on the role of cell-mediated immunity (CMI) (Sivanandan and Maheswaran, 1981; Sharma and Lee, 1983). Chickens in the age group of 3 to 6 weeks are the most susceptible to IBD infection (Fadly and Nazerian, 1983). It was observed that immunosuppression was maximum 48 h after experimental infection, though the infection was found to be established already 24 h after IBD infection and continued even beyond 72 h after IBD infection (Suresh, 1994). Hence, studies on IBD were conducted 48 h after experimental IBD infection in this work. In coccidiosis caused by *Eimeria* spp., the intestinal immune system of infected birds and its cellular components are directly involved in the transport of *E. tenella* sporozoites (Lawn and Rose, 1982; Lillehoj and Trout, 1993). The NK cell like activity of iIELs was found to be transiently suppressed in the early phase of primary infection, but enhanced during the post-patent period following eimerian infections (Lillehoj, 1989). Hence, studies on *E. tenella* were conducted 8 h after experimental infection with eimerian oocysts.

In the present study it was interesting to observe that the cytotoxic activity of iIELs was significantly depressed in chickens 48 h after experimental infection when compared to their age-matched healthy controls (significant at $p < 0.01$). These findings suggest a possible involvement of intestinal T cells in IBD infection through a mechanism which is difficult to explain from the present study. This depressed cytotoxic activity of iIELs might be due to loss of functional activity of iIELs while eliminating the viral antigens or due to the establishment of IBD infection in chickens, thereby implicating the gut (local) cell-mediated immunity. Conversely, the cytotoxic activity of iIELs was found to be restored in chickens previously sensitised with ISMP (significant at $p < 0.01$), followed by experimental IBD infection. However, BMCS failed to restore the cytotoxic activity of iIELs in birds primed with BMCS and experimentally infected with IBDV. Following primary infection with 10^5 oocysts of *E. tenella*, the cytotoxic activity of iIELs was found to be significantly depressed in chickens 8 h after *E. tenella* infection, when compared to their age-matched healthy controls (significant at $p < 0.01$). This decreased cytotoxic activity of iIELs was predicted to be due to the presence of some immunoregulatory iIELs (Lillehoj, 1989) or may be due to loss of iIELs activity in *E. tenella* infection as the iIELs

have been found to be directly involved in the transport of *E. tenella* sporozoites (Lawn and Rose, 1982). Prior sensitisation of chickens with immunostimulants such as ISMP and BMCS significantly contributed to restoring the cytotoxic activity of iIELs in *E. tenella* infection (significant at $p < 0.01$), although the former appeared to be a more potent immunostimulant (Figs 1 and 2).

It is well established that ISMP acts as a good nonspecific immunostimulant in different animal species (Mallick et al., 1985; Tewari et al., 1996). The complex nature of *Mycobacterium* cell wall and their heat shock proteins (HSPs) stimulate different cellular components of the immune system, activate them to secrete a number of cytokines, thereby augmenting the host's immune status (Mallick et al., 1985; Beagley et al., 1993). Optimal restoration of the cytotoxic activity of iIELs was found in ISMP-treated subgroups of IBD and *E. tenella* infected chickens, indicating the usefulness of this immunostimulating agent in restoring the immune status of the host in infections.

Experiments conducted by Lillehoj (1993) with a crude mixture of cytokines obtained from lymphocyte cultures suggested that lymphokines could immunologically regulate effector cells such as cytotoxic cells and NK cells to kill coccidian parasites. Similar results with BMCS further revealed that a crude mixture of cytokines could act as a potential immunostimulant in stimulating the host's immune response (Ghosal et al., 1990). Hence, bone marrow cultures were prepared from ISMP-treated birds and re-sensitised *in vitro* with ISMP to get a good yield of cytokines in BMCS and the same was used as an immunostimulant in BMCS-treated subgroups of IBD and *E. tenella* infected chickens. Studies on the effect exerted by immunostimulants ISMP and BMCS on the cytotoxic activity of iIELs during the postnatal development of chicks revealed that neither of these immunostimulants could modulate the functional activity of iIELs in normal, healthy conditions, i.e. when chicks were not infected with any infectious agent (Anand Kumar, 1995). This has confirmed the earlier findings that both immunostimulants (ISMP and BMCS) could modulate the host's immune responses only in infected states but not under normal and healthy conditions. These immunostimulants stimulate the host's immune system only if the host's immune responses are compromised, so as to overcome the infections. Hence, separate controls inoculated with ISMP alone and BMCS alone were not maintained in this experimental study. On comparative note, BMCS showed limited immunostimulating activity against primary *E. tenella* infection and practically no appreciable immunostimulating activity in IBD-infected chickens. As BMCS is a crude mixture of cytokines, it is difficult to surmise the involvement of cytokines in potentiating iIELs activity in *E. tenella* infection. Similarly, a careful analysis is required to find out which cytokines the BMCS apparently lacks which would be needed for potentiating the activity of iIELs in IBD infection. In the absence of specific information about the precise

mode of action of iIELs (Klein and Hamad, 1995) it is difficult to ascertain the precise mechanism involved in the impairment of iIELs activity in IBD infection. However, the incidence of orally transmitted secondary or concurrent infections in chickens with IBD gives a clue that some sort of iIELs activity is being impaired, breaking the first line of defence in the gut epithelium and increasing the susceptibility to secondary infections. As both IBD and coccidiosis are economically important diseases of poultry, application of combined immunostimulation seems to offer better prospects for the future. Further research is needed to find out the precise mode of action of iIELs in these infections, which seems to be quite promising.

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SUBPOPULATIONS OF LYMPHOCYTES IN CATTLE NATURALLY INFECTED WITH PAPILLOMAVIRUS

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Subpopulations of blood lymphocytes (CD2, CD4, CD8, WC1 and IgM-μ chain) were evaluated in clinically manifested bovine papillomatosis. Significantly lower numbers of CD2 (44.7%), CD4 (22.8%) and a lower ratio of CD4/CD8 (1.5) were found in animals with tumours compared to a group of cattle free of papillomas (62.3%, 34%, and 2.3, respectively). On the other hand, significantly higher numbers of gamma/delta+ T lymphocytes (9.6%) and of lymphocytes expressing IgM molecules (35%) were observed in the group of tumour-bearing cattle than in the papilloma-free group (4.8% and 22.1%, respectively). The animals came from a region characterised by elevated concentrations of copper, zinc, arsenic, cadmium and lead in the soil and in the organs of animals. It is supposed that environmental factors predisposed the animals to the development of papillomas.

Key words: Cattle, subpopulations of lymphocytes, papillomatosis, immunosuppression, flow cytometry

Skin papillomatosis of animals is a benign tumour disease of epithelial origin. Most papillomas develop as a result of infection by papillomavirus belonging to the Papovaviridae family. To date, six different types of papillomavirus have been identified as bovine papillomaviruses (BPV) 1–6 (Jarrett et al., 1991). Skin papillomas are very often induced by type BPV-2. Infection by this type occurs mostly through skin traumas and persists for about one year. Such changes were observed in both immunosuppressed and immunocompetent animals. The tumour, after its initial progressive growth in an affected animal, proceeds to spontaneous regression in the course of one year. This suggests that the regression of the tumour is caused by cell-mediated immunity (Jubb et al., 1993). There are no papers which define lymphocyte subpopulations in cows infected by papillomaviruses.

In this work, subpopulations of lymphocytes were determined with monoclonal antibodies against bovine lymphocytes in animals naturally infected with papillomavirus.

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Materials and methods

Animals. One- to four-year-old cattle (heifers and bulls of the Pinzgau breed) were divided into a control and an experimental group. The control animals (n = 8) were clinically healthy and without papillomatosis, while the experimental animals (n = 5) showed fibropapillomatosis. The animals came from an area located near a copper and zinc factory in the Spiš region.

Monoclonal antibodies. Monoclonal antibodies (MoAbs) were obtained from the International Laboratory for Research on Animal Diseases (ILRAD, Kenya). The monoclonal antibodies (Howard et al., 1991) used are summarised in Table 1.

Table 1

Monoclonal antibodies used

Specificity	MoAbs	Isotype	Dilution
CD2	IL-A42	IgG2a	1 : 1000
CD4	IL-A12	IgG2a	1 : 2000
CD8	IL-A51	IgG1	1 : 5000
gamma/delta+ T lymphocytes (WC 1)	IL-A29	IgG1	1 : 1000
IgM (μ chain)	IL-A30	IgG1	1 : 2500

Isolation of lymphocytes. Venous blood samples were collected from all animals and put into ethylenediamine tetra-acetic acid (EDTA). Lymphocytes were separated by sedimentation on Ficoll-Hypaque gradient (Boyum, 1974).

Procedure for flow cytometry. After separation, the lymphocytes were washed twice with phosphate buffered saline (PBS) supplemented with 0.2 per cent sodium azide and once in immunofluorescent medium (RPMI 1640 supplemented with 5 per cent fetal calf serum and 0.2 per cent sodium azide). Fifty μl of cell suspension (1×10^6 lymphocytes in immunofluorescent medium) and 50 μl of specific or control MoAbs were mixed and incubated at 4 °C for 30 min. After incubation the cells were washed twice in the immunofluorescent medium (IFM) and pellets were mixed with 25 μl of FITC-conjugated anti-mouse immunoglobulins and incubated as described above, in the dark. The working dilution of monoclonal and FITC-conjugated anti-mouse immunoglobulins (Institute of Sera and Vaccines, Prague, Czech Republic) was determined by titration and the calculation of a resolution index. After being stained the cells were washed twice in the IFM and once in PBS supplemented with 0.2 per cent sodium azide. The cells were resuspended in 0.5 ml of the same buffer.

Analysis of stained cells. The fluorescence-activated cell sorter (FACScan, Becton Dickinson) was provided with a 15 mW argon ion laser. The

analysis examined a dot plot and histogram of the leukocyte obtained by the forward and side scattering physical character of the lymphocyte population (Fig. 1). The results are therefore expressed as the percentage of the lymphocyte population which was positive for a specific MoAb. An unrelated MoAb of similar isotype was used as control.

Statistical evaluation. The unpaired Student's *t*-test was used for statistical analysis.

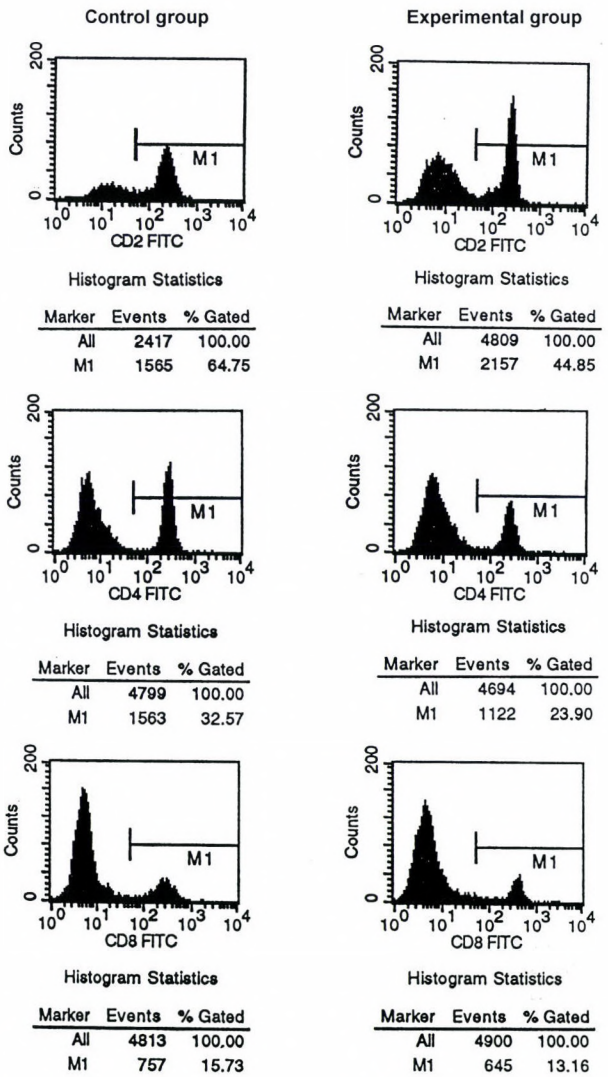

Fig. 1. Single-parameter histogram of bovine lymphocytes stained by fluorescein isothiocyanate (FITC)-conjugated anti-CD2, anti-CD4 and anti-CD8 MoAbs

Results

In the experimental group of animals a decrease of CD2 T lymphocytes (44.7%) was recorded compared with the control animals (62.3%). The significance was on the level of $P < 0.001$ (Table 2). Likewise the decrease of CD4 lymphocytes (22.8%) was registered in the experimental group compared with the control group (34%), with a significance of $P < 0.05$. No change in CD8 cell numbers was found between these groups. The CD4/CD8 ratio was 1.47 in the experimental group and 2.32 in the control group. On the other hand, an increase of gamma/delta+ T lymphocytes (9.6%) was recorded in the experimental animals, compared with 4.8% in the control group (significance of difference: $P < 0.001$). An increase of cells producing IgM was also found in the experimental group compared to the control group, with a significance of $P < 0.05$.

Table 2

Evaluation of lymphocyte subpopulations by FACS

Lymphocyte subpopulations	Experimental group (n = 5) (%)	Control group (n = 8) (%)
CD2	44.662 ± 2.105^{c}	62.331 ± 2.422
CD4	22.768 ± 1.246^{b}	33.999 ± 1.875
CD8	15.880 ± 1.412	16.010 ± 1.893
gamma/delta+ T lymphocytes	9.558 ± 0.954^{c}	4.832 ± 0.563
IgM (μ chain)	35.158 ± 3.560^{a}	22.145 ± 2.848
CD4/CD8	1.472 ± 0.322^{a}	2.321 ± 0.701

Statistical significance on the level: $^{a}P < 0.05$, $^{b}P < 0.005$, $^{c}P < 0.001$

Discussion

In the experimental animals, numerous and unusually widespread fibropapillomas, some of enormous dimensions, were observed (Švrček et al., 1995). The virus was demonstrated in these papillomas with a polyclonal antibody against a group-specific antigen of bovine papillomavirus immunohistochemically (Levkut et al., 1997), and by electron microscopy (Zibrín et al., unpublished). The tumours from cows were in the regressive phase of growth at venepuncture for the determination of lymphocyte subpopulations (Levkut et al., 1997).

There were about 22% fewer CD2 lymphocytes in the experimental group than in the control group. It is known that the majority of T lymphocytes in cows

display the CD2 molecule (rosette receptor). For the most part CD2 lymphocytes also possess the antigens CD4 and CD8 (Tizard, 1992). In our experiment a decrease in the percentage of CD4 lymphocytes was found, while the number of CD8 lymphocytes resembled that of lymphocytes in the control group. As a result, the change of the CD4/CD8 cell ratio in the experimental group was at the expense of the CD4 cells. The predominance of CD8 cells over CD4 cells indicates considerable suppression by CD8 cells. Likewise, increased suppression can be observed in animal diseases which cause immunosuppression, such as infection by feline immunodeficiency virus (Peri et al., 1994).

The isolated circumscribed perivascular infiltration of the derma of papillomas by CD3 cells in this period of growth has a considerable immunosuppressive effect in animals with papillomas, together with conspicuous multiplication and release of the virus into the cell cytoplasm and extracellular matrix already in the stratum spinosum of the papilloma's growth (Zibrín et al., unpublished). In immunocompetent animals, maturation and release of the virus into the cytoplasm and extracellular matrix take place in the stratum corneum of the epidermis (Olson, 1990).

There was a considerable increase in the number gamma/delta+ T lymphocytes in the experimental animals as opposed to the controls. These lymphocytes are known to have a tendency to associate with epithelial cells. The primary location of these cells is in the skin close to the stratum basale. They are distinguished by their skin alloantigens, and can be cytotoxic (Tizard, 1992). It can be said that generally a low number of gamma/delta+ T lymphocytes exists in both groups, which can be caused by factors in the animals' environment (Campo, 1987). The area studied in our experiment is characterised by elevated levels of copper, zinc, arsenic, cadmium, and lead in the soil and in animals (Vrzgula et al., 1986). Bíreš et al. (1995) demonstrated the negative influence of these metals on the health, immune response, production, and reproductive efficiency of sheep.

The significantly higher number of B lymphocytes (IgM expression) relates to the enhanced stimulation of B cells which proliferate in animals with papillomas. Many authors, however, state that the consequent expression of papillomas is not related to the production of serum antibodies but probably to T-cell immunity (Olson, 1990).

The significantly lower numbers of T lymphocytes and the low CD4/CD8 ratio found in the experimental animals with respect to the controls relate to cellular immunosuppression in infected animals. The results support the suggestion that the regression of tumours is caused by cell-mediated immunity. The development of papillomas in the region investigated suggests that external factors can markedly affect the immunological status of animals.

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ERADICATION OF BRUCELLOSIS FROM A CATTLE HERD IN THE AMAZONIAN REGION (SHORT COMMUNICATION)

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The authors intend to elaborate a brucellosis eradication program which could be used in the Amazonian region, an area characterised by extremely extensive animal husbandry practices. Under such conditions, eradication by selection is the only feasible approach. Brucellosis has been successfully eradicated from a herd with 22.1% prevalence of infection by two serological surveys using an indirect ELISA and the complement fixation test.

Key words: Brucellosis, eradication, cattle, serology, Amazonian region

In the majority of the highly developed countries, *Brucella abortus* infection has been successfully eradicated from cattle herds or its prevalence has been markedly reduced. The remaining infected herds have been drawn under strict surveillance so that they can either be rendered free of infection or culled as soon as possible. At the same time, despite the intensification and concentration of production, *B. abortus* infection is still spreading in some of the developing countries.

The Brazilian part of the Amazonian region has a cattle population of about 15 million. Most of those animals are kept under extensive conditions. The calves stay on the pasture throughout their life and are weaned at 6–8 months of age. Depending on the quality of pasture, their feeding may be poor, medium-quality or good; accordingly, the calves reach slaughtering weight (400–500 kg) in 2 to 8 years. The use of fodder salt, of powder form and consistent composition, has become widespread in recent years. Cattle do not receive any other feed supplement in most cases. The size of the dairy cattle population is very small in the region; thus, towns are supplied with milk and dairy products by dairy farms located at a distance of 2,000–3,000 kilometres, in the central and southern part of the country. No reliable data are available on the incidence and prevalence of infectious diseases. The diagnosis of brucellosis is based on the buffered plate agglutination test, as follows:

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Serum dilution	Non-vaccinated animals	≥ 2-year-old animals vaccinated at 4–8 months of age
1:25	–	–
1:50	doubtful	–
1:100	+	doubtful
1:200	+	+

Even that serological test is carried out only in cases when this is stipulated by law (e.g. on animals to be transported to shows or auctions), or if the buyer requires the "brucellosis-free" status of the animals to be bought.

The majority of the herds were found to be infected by the serological tests carried out by us [indirect ELISA, complement fixation test (CFT), buffered plate agglutination test]. The rate of infection within herds varied between 5 and 53%.

Some farmers who wish to intensify production and, therefore, supply their animals with permanent identification, divide the vast pastures into smaller parcels of land, and have certain tests carried out to detect or rule out certain infectious diseases (the use of such tests is, however, commensurate with the usually rather narrow means of the farmers). Eradication attempts have been started in six such herds. In two of the herds only a single blood test was carried out: after having been informed of the results, the farmer gave up the idea of eradication. In three herds eradication is currently in progress while in one herd it has already been completed. This last eradication effort can be considered exemplary and, therefore, is reported here.

On the farm, one of the farmer's favourite horses developed fistulous withers. Based upon that lesion the veterinarian diagnosed brucellosis. Wishing to free his farm from brucellosis, the farmer asked the regional diagnostic institute to direct the eradication program. The above-mentioned plate agglutination test was carried out and gave the following results (Table 1).

Table 1

Results of the buffered plate agglutination test

Date of testing	Number of cattle tested	Positive		Number of horses tested	Positive	
		n	%		n	%
24.03.1995	365	23	6.3	43	6	14
22.06.1995	333	3	0.9	no testing was done		
20.09.1995	328	–	–	37	–	–

On the basis of the results the herd was declared free of brucellosis. On 20 October 1995, a cow aborted at an advanced stage of pregnancy.

The second eradication attempt was based upon the results of indirect ELISA (FAO/IAEA, Vienna) and CFT. The ELISA was carried out on the basis of the Manual of Standards for Diagnostic Tests and Vaccines (OIE, 1992) while the CFT according to the principles described in the relevant handbooks. The aggregate results of the two tests are presented in Table 2.

Table 2

Results of ELISA and complement fixation test (CFT) performed during eradication

Serial no. of the test	Date of testing	Number of cattle tested	Positive		Number of horses tested*	Positive	
			n	%		n	%
1	16.11.1995	303	67	22.1	no testing was done		
2	11.03.1996	204	11	5.4	38	7	18.4
3	05.06.1996	193	–	–	31	–	–
4	24.09.1996	193	–	–	31	–	–

*the serum samples of horses were tested only by CFT

There were major differences between the two tests in the number of positive results obtained (Table 3).

Table 3

Number of samples positive by ELISA and by the complement fixation test (CFT)

	First testing	Second testing
Number of samples positive by both tests	32	7
Number of samples positive only by CFT	1	–
Number of samples positive only by ELISA	34	4
Total number of seropositive samples	67	11

As far as we know, this has been the first successful attempt to eradicate brucellosis from a cattle herd in the Amazonian region. As had been expected, the above-described version of the plate agglutination test proved unsuitable for use in brucellosis eradication. Although the aetiology of the abortion that occurred on 20 October 1995 has not been elucidated with complete certainty, it was probably caused by *B. abortus* (one month after abortion the cow's serum was positive by both tests; abortion took place about 2–4 weeks before term).

The ELISA was much more sensitive than the CFT (Table 3). The higher sensitivity of the ELISA was undoubtedly one of the major factors contributing to the success of eradication. Perhaps the cut-off value (cut-off = 35) should have been set at a higher value, in which case less animals would have been seropositive even by the ELISA; however, we considered it better to remove two false seropositive animals than to leave a seronegative or serologically doubtful but infected animal in the herd. This view was shared by both the attending veterinarian and the owner.

The first nation-wide brucellosis eradication programmes were carried out in the Scandinavian countries. By the end of the 'sixties, the basic principles of versatile eradication programmes had been laid down (Morgan and McDiarmid, 1968) which were then adopted, with more or less modification, by technical books and manuals (Blood and Henderson, 1976; Beer, 1988).

Brucellosis can theoretically be eradicated from infected herds by selection, generation shift, and herd replacement. Eradication by selection can usually succeed only if the number of positive animals detected by all serological tests does not exceed 10%, and if no abortion occurs in the given herd. However, in the circumstances and under the farming conditions outlined above eradication can be achieved exclusively by selection. Some of the local conditions favour that eradication approach while others act against it. One of the conditions favouring the eradication is that in the majority of cases vast expanses of land are available. By dividing these areas into smaller parcels, several groups of animals can be isolated, and even animals belonging to the same group can disperse in a large area. At the same time, eradication is hampered by the, sometimes total, lack of knowledge and discipline. In addition, cattle are not marked in a permanent manner in the majority of herds, which makes it very difficult to single out positive animals from large groups of cattle and to isolate them from their negative herdmates in a reliable manner.

As regards the chances of successful brucellosis eradication, we can say that satisfactory work discipline, the use of sensitive serological tests, and the strict and rapid removal of seropositive animals could soon lead to success also in the highly infected herds. (In the case presented here, the old seronegative cows of no particular value were also removed from the herd after the first test.)

It is worth mentioning that the conception rate rose from about 60% to approx. 90% in the herd.

Animals from the outside may be introduced into the herd rendered free of brucellosis only if they have calved at term and if their blood samples taken 2–3 weeks after calving have given negative results by ELISA and CFT. If heifers are purchased, they should be subjected to two serological tests before calving.

It is a very important question how long the brucellosis-free status of the herd can be maintained in the highly infected environment. We would like to

implement the "regionalisation principle", i.e. to eradicate the infection from all cattle and buffalo herds, as well as from all other animal species (horses, dogs, cats) that may play an important role in the transmission of *B. abortus* within a given region.

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PROTECTION OF BROILER BREEDERS BY AN INACTIVATED COMBINED WATER-IN-OIL-IN-WATER VIRAL VACCINE

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A four-component vaccine, prepared by combining the single vaccines, contains subunits of Newcastle disease and infectious bronchitis viruses, as well as whole inactivated infectious bursal disease and egg drop syndrome viruses. The vaccine is prepared in the form of a low-viscosity water-in-oil-in-water emulsion with low mineral oil content. Heavy breeders were vaccinated at the age of 20 weeks by intramuscular administration of 0.5 ml vaccine/bird in an experiment carried out under field conditions, involving 5000 female and 450 male parents. The birds had previously been vaccinated with live vaccines according to an obligatory field vaccination programme. Vaccination with the WOWE vaccine near the point of lay elicited serological responses protecting both the parents and their progeny. Each of the antigens administered in the four-component vaccine was as effective as the respective single component vaccine. The mortality, recorded during the 31-week experimental period, was 6.2%. Mortality and morbidity were not triggered by viruses against which vaccination was carried out. Egg production was not affected by the vaccination and was 170.2 eggs per hen during the 28-week production period.

Key words: Combined vaccine, egg drop syndrome, egg production, heavy breeders, inactivated vaccine, infectious bronchitis, infectious bursal disease, Newcastle disease, maternal antibodies, poultry vaccine

A typical programme for the protection of replacement breeders against Newcastle disease (ND), infectious bronchitis (IB) and infectious bursal disease (IBD) includes one or a few vaccinations with live viruses and a revaccination with inactivated viruses near the point-of-lay (Glisson and Kleven, 1993). Widely used vaccination programmes against egg drop syndrome (EDS) comprise a vaccination with inactivated viruses prior to the laying period. Consequently, an inactivated vaccine comprising four virus species may be used for the vaccination of breeders near the onset of egg production. In such a way, vaccination expenses and losses caused by vaccination stress could be significantly

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reduced. The currently available inactivated combined poultry vaccines contain antigens such as ND, IBD, EDS and IB viruses and reoviruses. The efficacy of inactivated poultry viral vaccines is usually increased by the addition of light mineral oils. The use of multiple water-in-oil-in-water emulsions (WOWE) eliminates the undesirable side effects which accompany the application of mineral oil adjuvanted vaccines.

The main objective of the work presented in this paper was to evaluate the serological immune response of broiler breeders to a combined inactivated vaccine containing ND, IB, IBD and EDS viruses. The birds had previously been vaccinated with live vaccines. Vaccination with the combined vaccine was carried out prior to the beginning of lay. The combined vaccine was prepared in the form of a low-viscosity WOWE and the oil- to water-phase ratio was two- to eight-fold lower than that reported for experimental poultry vaccines (Stone et al., 1981; Stone, 1988, 1991). This vaccine differs from other inactivated poultry vaccines since the ND and IB viruses are previously inactivated and solubilised by the non-ionic detergent Tween 80. Tween 80 is also used as a water-phase emulsifier. The treatment of enveloped viruses with a detergent results in the release of the antigenic surface proteins. Partial replacement of whole viruses by their subunits in a combined vaccine is the first step towards the production of ecologically convenient and inexpensive vaccines based solely on the viral subunits.

Materials and methods

Birds and vaccination

The primary vaccination of 5000 female and 450 male Ross broilers was carried out by the administration of live lentogenic or attenuated viral strains. The birds were vaccinated once against Marek's disease (Marikal, Pliva, Zagreb, Croatia), IBDV (Gumbokal, Pliva), fowlpox (Bodikal, Pliva) and avian encephalomyelitis (TAD, Cuxhaven, Germany), and twice against NDV (Pestikal – LaSota, Pliva) and IBV (Bronhikal, Pliva). Marek's disease vaccine was administered intramuscularly (i.m.) at the back of the neck of one-day-old chickens. At the age of 10 days, the chickens received the vaccine against IBDV via the drinking water. The live vaccine against fowlpox was administered by the wing-web method at the age of 8 weeks, while the vaccine against avian encephalomyelitis was given via the drinking water at the age of 15 weeks. At the age of 3 and 8 weeks, chickens were spray-vaccinated against NDV. The H-120 strain of IBV was administered by the coarse spray method and via the drinking water at the age of 1 day and 4 weeks, respectively. At the age of 20 weeks, birds were inoculated i.m. with 0.5 ml/bird of an experimental WOWE viral vaccine com-

posed of four one-component vaccines. At the same time, single one-component vaccines were administered to four groups of 25 birds each. The form and content of viral antigens in the one-component vaccines were the same as in the combined vaccine. Two control groups, one formed of the female and another of the male birds, containing 25 birds each, were not vaccinated with the experimental WOWE vaccine and were kept in separate boxes.

Eggs, collected 31 weeks after the vaccination of dams, were incubated and hatched. A group of 25 hatched chicks was analysed for the presence of maternal antibodies. Blood samples were taken from 3- and 10-day-old chicks.

During the rearing period, birds were fed nutritionally adequate diets and treated according to the producers' recommendations.

Combined four-component experimental vaccine

LaSota strain of NDV (Pokrić et al., 1993), Winterfield 2512 strain of IBDV, M 41 strain of IBV and 127-like strain of EDSV were propagated in eggs. Viruses were inactivated with 0.1% v/v formaldehyde. After accomplishing NDV and IBV inactivation, viruses were solubilised by the non-ionic detergent Tween 80 (polysorbate 80) (Čajavec et al., 1996). Safety testing, carried out on embryonated eggs, proved that the inactivation of IBDV and EDSV, as well as the inactivation and solubilisation of NDV and IBV, completely abrogated the infectivity. The amount of viral material incorporated in a vaccine dose, corresponding to non-inactivated viruses, amounted to $1 \times 10^{8.5}$ 50% embryo infective dose (EID_{50}) of NDV, $1 \times 10^{6.7}$ EID_{50} of IBV, $1 \times 10^{6.5}$ EID_{50} of IBDV, and $1 \times 10^{8.5}$ EID_{50} of EDSV. Each of the immunogens was individually prepared as a single vaccine in a double WOWE (Čajavec et al., 1996). The WOWE contained Tween 80 as the water-phase surfactant. The oil-phase was composed of the light mineral oil Bayol supplemented with the oil-soluble surfactant Arlacel 83 (sorbitan sesquioleate). Water- and oil-phases were mixed in a proportion of 2:1. The actual concentration of both oil-phase Arlacel 83 and water-phase Tween 80 surfactants in the vaccine was 3.3% w/v. Taking into account the ratio of surfactants (1:1) and the hydrophile-lipophile balance (HLB) values of Arlacel 83 (HLB = 3.7) and Tween 80 (HLB = 15), the HLB value of the surfactant mixture was 9.35.

The integrity of the emulsion was controlled by a drop test in a manner described in details elsewhere (Herbert, 1978).

The relative viscosity of the WOWE vaccine (n = 3.45) was obtained from the ratio of flow times of WOWE and distilled water (mean of 10 determinations), determined in Ostwald's viscosimeter at 20 °C.

One-component vaccines were prepared by mixing one volume part of stock vaccine with three volume parts of WOWE. The combined vaccine was

obtained by mixing the equal volumes of stocks of four individual WOWE one-component vaccines.

Serology

Blood samples were taken by brachial vein puncture. In order to take serum samples always from the same birds, 25 birds from each of the pullet and cockerel flocks were wing-banded prior to blood collection. The separation and inactivation of blood sera were described in detail elsewhere (Pokrić et al., 1993). The levels of antibodies against NDV were determined by both HI and ELISA (FlockCheck®, IDEXX Corp., Portland, ME, USA) tests. The levels of antibodies against IBV and IBDV were determined by ELISA (FlockCheck®), while antibodies against EDSV were analysed by the HI test. In order to determine the levels of antibodies against both NDV and IBV, 4 HA units of viruses were used in the HI tests. The ELISA tests were carried out and the ELISA titres were calculated according to the manufacturer's instructions (FlockCheck Production Guide). The ELISA titres are presented as the experimentally obtained values divided by 1000.

Egg yolks, taken for the determinations of ELISA titres of antibodies against NDV, IBV and IBDV, were separated from the eggs collected 20, 25 and 31 weeks after the vaccination of layers with the experimental vaccine. Fifteen eggs were taken for each analysis. Yolks were diluted four times in phosphate-buffered saline (PBS) and analysed according to the protocols for serum analyses. Each sample was analysed twice.

Morbidity and mortality

Morbidity and mortality were recorded daily. The dead birds were necropsied and organs were submitted for pathological analysis.

Results

The levels of specific antibodies in the broiler parents vaccinated against NDV, IBV, IBDV and EDSV are presented in Figs 1 and 2. Each HI titre value in Fig. 1 represents an arithmetic mean of $\log_2$ values determined in duplicate in serum samples taken from 25 birds. Each experimental point corresponding to the ELISA titres is an arithmetic mean of the titres determined in duplicate in serum samples taken from 25 birds (Fig. 2).

Twenty-week-old pullets and cockerels primed against EDSV by a four-component WOWE vaccine developed a significant level of antibodies as soon as two weeks postvaccination (Fig. 1). Antibody titres reached the peak between

the fourth and the sixth week postvaccination, and then gradually fell during the subsequent 15 weeks. A second increase of antibody levels was observed 25 weeks postvaccination, followed by a decrease until the end of the experiment. Comparable immune responses against EDSV were elicited by vaccination with both four- and one-component vaccines. No EDSV antibodies were detected in the control groups which had received no vaccine.

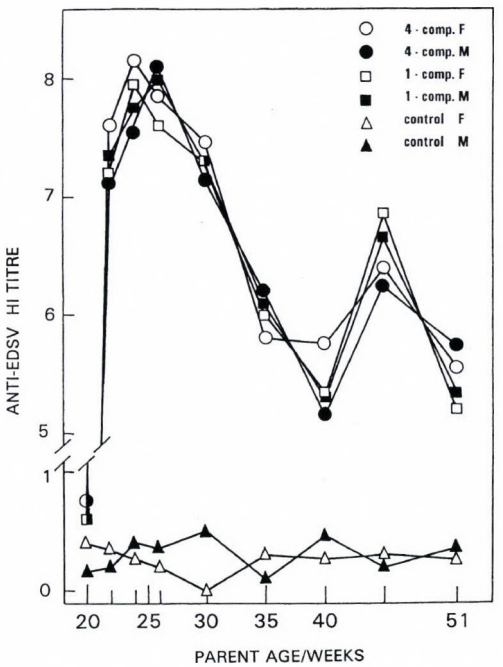

Fig. 1. Serological response of female (F) and male (M) broiler parents receiving EDSV in one- (1-comp.) and four-component (4-comp.) vaccines at the age of 20 weeks. The vaccines were prepared in the form of WOWE. Control groups were not vaccinated

More significant variations in the levels of antibodies during the experimental period were observed after the revaccination of female and male parents against NDV and IBV rather than after IBDV revaccination (Fig. 2). However, a similarity between the patterns of humoral immune response elicited by all four antigens in both females and males, either by the primary vaccination or by the revaccination, was evident (Figs 1 and 2). Since a good correlation factor ($r > 0.8$) was obtained between immune responses generated by viral antigens administered in the form of a four-component and a one-component vaccine (Fig. 1), data concerning immune responses against NDV, IBV and IBDV administered in the form of single vaccines are not presented in Fig. 2.

The hens started to lay at the age of 23 weeks, i.e. 3 weeks after vaccine administration (Fig. 2). Maximum egg production was recorded 10 weeks post-vaccination. During the production period of 31 weeks, average egg production per hen was 170.2 eggs of an appropriate quality. The levels of specific antibodies in the egg yolk were at least twice higher than those in the sera of dams. Maternal antibody levels in the sera of chicks, hatched from eggs collected 31 weeks after the vaccination of dams, significantly declined in the period between the third and the tenth day of life (Fig. 2). The levels of specific antibodies in the non-vaccinated controls continuously decreased and reached negligible values by the end of the experiment (data not presented in Fig. 2).

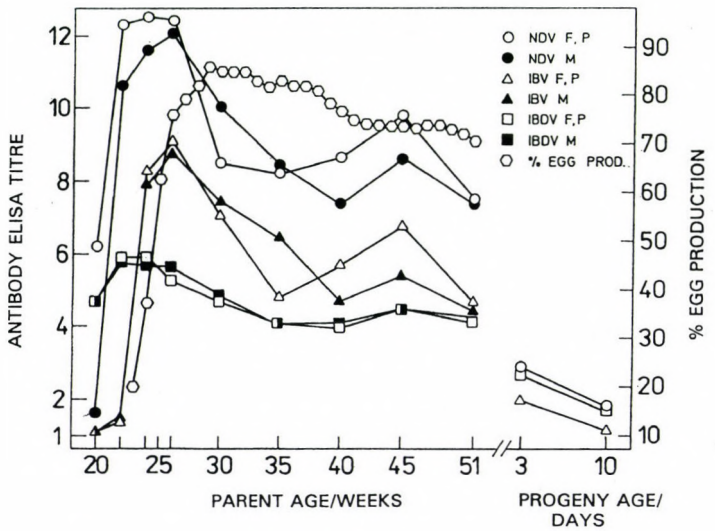

Fig. 2. Egg production and serological response (ELISA titres) of female (F) and male (M) broiler parents against NDV, IBV and IBDV after revaccination with a four-component WOWE vaccine at the age of 20 weeks. The levels of specific maternal antibodies were also measured in sera of progeny (P) hatched from 51-week-old breeders. The ELISA titres are presented as experimentally obtained values divided by 1000. Since a good correlation ($r > 0.8$) was obtained regarding the immune responses generated by a viral antigen administered in one-component vaccines, data concerning immune responses against respective single vaccines are not presented. So far, no differences have been detected between patterns of humoral immune response against NDV as determined by ELISA and by HI, respectively; thus, HI titres are not presented

The total mortality recorded over a 31-week period after vaccine administration was 6.2%. Clinical and pathological examinations proved that neither morbidity nor mortality could be attributed to ND, IB, IBD or EDS.

Discussion

The four-component vaccine applied in this study in a manner recommended for the vaccination of hens prior to the onset of egg production (Eidson et al., 1980; Gough et al., 1981; Eidson et al., 1982; Zanella and Marchi, 1982; Thayer et al., 1983, 1983*a*, 1983*b*; Box, 1992; Glisson and Kleven, 1993) generated significant levels of specific NDV and IBV antibodies in the parents and maternally derived antibodies in the progeny (Fig. 2). It is important to emphasise that this is the first time that a vaccine containing subunits of ND and IB viruses was applied for the vaccination of heavy breeders near the onset of lay. Satisfactory immunogenicity of the subunits, despite the low amount of viral antigens in a vaccine dose, results from the controlled treatment of viruses with the non-ionic detergent. The detergent solubilises the viral membranes, causing the release of antigenic envelope proteins (Pokrić et al., 1993; Pokrić and Čajavec, 1995). The antigenic surface proteins required for the generation of humoral immune responses are the HN and $F_{1,2}$ glycoproteins of NDV, as well as the S_1 and S_2 glycoproteins of IBV (Meulemans et al., 1988; Boursnell et al., 1990; Boursnell et al., 1990*a*; Nishimo et al., 1991; Collisson et al., 1992; Ignjatovic and Galli, 1995; Kwon and Jackwood, 1995). Both the detergent and the experimental conditions chosen favoured the formation of aggregates of the surface glycoproteins (Schied and Chopin, 1973; Pokrić et al., 1993). Aggregates of higher molecular mass are more effective than glycoprotein monomers in eliciting an immune response. The glycoproteins released after delipidation of the membrane by a mild non-ionic detergent and bound in the aggregates preserve membrane orientation (Lichtenberg et al., 1983). This preserves glycoprotein conformation and also contributes to the immunogenicity of the aggregated glycoproteins.

The purpose of vaccination against IBDV was not the protection of breeders. Older birds were not exposed to IBDV. The vaccine generated a uniform level of antibodies which, upon transfer to the progeny, could ensure passive protection during the first days of life (van der Berg and Meulemans, 1991; Lasher and Shane, 1994). Young birds were not exposed to EDSV and, therefore, a maternally-derived immunity against EDSV was not determined.

Our vaccine was prepared in the form of WOWE, since double WOWEs have proven to be as antigenic as single oil-in-water or water-in-oil emulsions, while being much less viscous and more stable for inoculation (Droual et al., 1990). The low content of mineral oil and the appropriate ratio of oil- and water-surfactants in our WOWE contributed to the low viscosity of the vaccine. Therefore, the vaccine has been found to be very suitable for injection. None of the side effects that accompany the use of oil-adjuvanted vaccines, such as tissue lesions (Yamanaka et al., 1993), were detected at the site of injection of our vac-

cine. The WOWE formulation could also account for the second rise of specific antibody levels seen at a later stage of the laying period (Figs 1 and 2). This was observed by us in numerous field experiments when killed WOWE vaccines were administered near the onset of lay. We suggested that this second rise of antibody levels could be associated with the immunoendocrine factors elicited by an increase of prolactin levels during the laying cycle (Čajavec et al., 1996). However, the lack of difference in the pattern of humoral immune responses between hens and cocks (Figs 1 and 2) directs attention to the influence of the vaccine formulation rather than the immunoendocrine factors. The presence of Tween 80 in the vaccine contributes to a high HLB value resulting in increased dispersibility of the water-in-oil emulsion. This leads to a continuous and increased rate of release of the viral antigens from the water phase (Stone et al., 1983; Stone, 1988), and may act as a repetitive booster effect which elicits the repeated enhancement of humoral immune response.

From the cost-benefit point of view, our combined vaccine prepared from the individual single vaccines proved to be very convenient. A single revaccination before the laying period was sufficient to protect both replacement and infection-exposed progeny flocks against field challenge (Allan et al., 1978; Mockett et al., 1987; Giambrone and Closser, 1990; van Eck, 1990; Glisson and Kleven, 1993; FlockCheck Production Guide, 1991). No additional vaccinations, which could influence the egg production, are required. The combined vaccine retained the dose volume of 0.5 ml and produced a humoral immune response to all four antigens indistinguishable from that produced by either vaccine in a one-component form. The replacement of a few vaccinations with the individual vaccines by a single administration of the combined vaccine significantly reduces the cost of immunoprophylaxis and the production losses caused by vaccination-related stress.

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SONOGRAPHIC DIAGNOSIS OF INTESTINAL OBSTRUCTION IN THE DOG

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Ultrasonography was performed on 44 dogs to decide whether small bowel obstruction was present. The sonographic criteria for small bowel obstruction were (1) the presence of pendulous movement of the ingesta inside the dilated bowel, (2) observation of invaginated intestines or an ingested intraluminal foreign body, (3) observation of non-uniform peristaltic activity of the dilated intestines, or (4) observation of akinetic intestinal loops together with abdominal fluid accumulation. By using these criteria, obstruction was correctly diagnosed by ultrasonography in 11 of the 13 dogs with mechanical ileus, and obstruction was correctly excluded in 29 of the 31 non-obstructive cases. Thus, the above-mentioned sonographic criteria had 85% sensitivity and positive predictive value, and 94% specificity and negative predictive value. The present study suggests that ultrasonography is a valuable tool for diagnosing small intestinal obstruction in the dog.

Key words: Dog, ileus, intestinal diseases, small bowel obstruction, ultrasonography

Partial or complete obstruction of the small intestine of the dog can be caused by indigestible foreign material, masses of parasites, postoperative adhesions, neoplasm, granulomas, abscesses, volvulus, intussusception and hernial incarceration. Paralysis of a segment or the entire small bowel caused by peritonitis, enteritis, pancreatitis, certain drugs, or following laparotomy may cause signs of intestinal obstruction (Fraser, 1991). Moreover, intestinal paralysis can also be a result of a prolonged mechanical obstruction. Intestinal obstruction can be a surgical emergency; thus, differentiation of surgical cases from those that can be managed by means of conservative treatment is of primary importance when evaluating a dog with signs of ileus. The diagnosis is traditionally based on the physical findings and proven by plain film radiography. If the physical and radiographic findings are equivocal, repeated films are taken following the administration of a radiographic contrast material. The sensitivity of the contrast examination (upper gastrointestinal study) is low and enteroclysis has been proposed as a sensitive tool to diagnose those cases where the previous radiographic findings are in-

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conclusive (Wolvekamp, 1989). However, this latter technique requires the use of fluoroscopy, which is not readily available in veterinary medicine.

Utrasonography has been used for many years to diagnose the disorders of various abdominal parenchymal organs in the dog. The sonographic appearance of the normal canine gastrointestinal tract and that of some gastrointestinal disorders have also been described (Penninck et al., 1989, 1990). Reports on the sonographic appearance of canine intestinal ileus are limited to that of gastrointestinal foreign bodies, invagination and paralytic ileus (Fluckiger and Arnold, 1986; Kantrowitz et al., 1988; Penninck et al., 1990; Watson et al., 1991; Tidwell and Penninck, 1992; Kramer and Gerwing, 1996). Numerous authors have described the sonographic findings of small intestinal obstruction in human beings. There are also sonographic criteria for the diagnosis of this disorder in humans (Ko et al., 1993; Ogata et al., 1994, 1996; Truong et al., 1992). The aim of this paper was to establish similar sonographic criteria, and evaluate their efficacy in the diagnosis of intestinal obstruction of the dog.

Materials and methods

Between 1 May 1996 and 30 April 1997, all dogs that were presented to the Department and Clinic of Internal Medicine at the University of Veterinary Science Budapest, and were determined to have possible small bowel obstruction on the basis of the clinical examination, were candidates for this study. Patients with the signs of intestinal obstruction (vomiting, abdominal pain, abnormal findings of abdominal palpation, changes in defecation) were entered into this study only when a sonographer experienced in the technique of intestinal imaging was available at the time of patient evaluation. Altogether 44 dogs underwent abdominal ultrasonography as part of their routine diagnostic work-up using commercially available ultrasound scanners equipped with 3.2, 5 or 7 MHz sector transducers (Bruel & Kjaer 1640 and Bruel & Kjaer Panther 2000, Naerum, Denmark). No particular preparation was given to the dogs other than clipping the hair from the ventral abdomen, and application of ultrasound gel. The interference by gas echoes from the bowels was avoided by changing the dogs' position and scanning from different planes as described by Penninck et al. (1989). Sonographic findings were recorded on VHS videotape during the scanning, and the reports were stored in a computerised patient data system immediately after the examination. In the sonographic report particular attention was paid to the presence of intestinal obstruction. The sonographic diagnosis was established by using previously determined criteria based on the authors' former experience. These criteria for small intestinal obstruction were: (1) the presence of one or more fluid-filled small intestinal loop(s) with unsuccessful

peristaltic activity, observed as a pendulous, i.e. "to-and-fro" movement of the intestinal ingesta; (2) the presence of invaginated intestinal loops or a foreign body which transmits the ultrasound beam in the distended bowel; (3) distended small intestinal loops with non-uniform peristaltic activity (both increased and decreased); or (4) the presence of akinetic intestinal loops together with free abdominal fluid accumulation in the abdomen. If any of these signs was present, the sonographic diagnosis of intestinal obstruction was made (Figs 1 and 2).

The sonographic criteria for paralytic ileus were the observation of fluid-filled intestinal loops with decreased or no peristaltic activity, and without the above-mentioned signs of an obstruction (Fig. 3).

When examining a dog with non-distended intestines, or with moderately distended intestines and uniformly increased peristalsis, we considered the case not having any forms of ileus.

Abdominal surgery was performed in all dogs where sonographic signs of intestinal obstruction were present. In dogs with paralytic ileus, or without the sonographic signs of ileus, laparotomy was performed only if other investigations (radiography) indicated long-standing obstruction or perforation. The final diagnosis was established on the basis of the result of surgery, post-mortem examination, or the clinical outcome of the case. The sonographic findings were compared with the final diagnoses in order to determine the sensitivity, specificity, positive and negative predictive value of the above-mentioned criteria (Rijnberk, 1995).



Fig. 1. Cross-sectional ultrasound image of invaginated intestinal loops in a dog. The outer circle represents the intussuscipiens, the inner circle the intussusceptum. Between the two intestinal segments, entrapped mesentery and dilated vessels are also visible

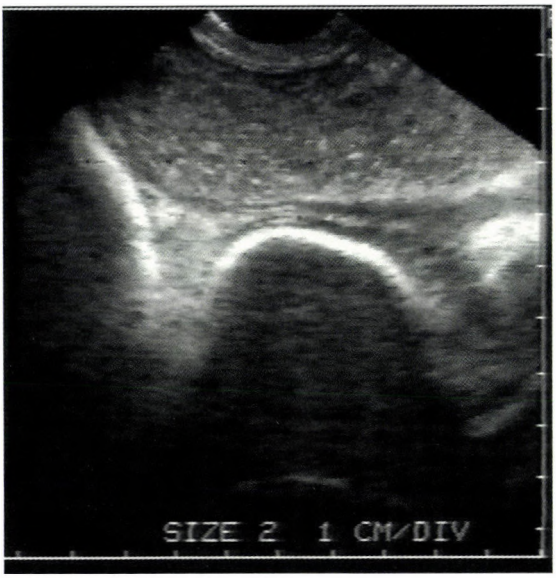

Fig. 2. Ultrasound image of a dilated small intestinal loop in longitudinal section. The luminal content of the intestine is highly echogenic. Under this intestine the echogenic reflexes and acoustic shadowing caused by the gas content of other intestinal loops are also visible. Pendulous movement of the ingesta could be observed in the fluid-filled intestine during the examination. Ultrasound diagnosis was intestinal obstruction

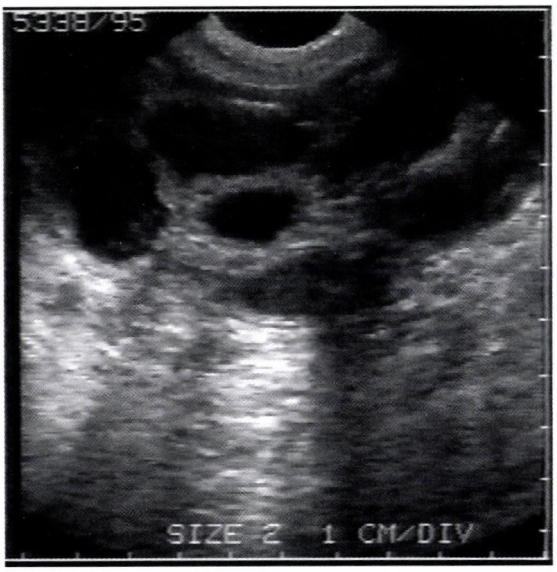

Fig. 3. Dilated fluid-filled intestines in a dog (cross section). No peristalsis was visible during the examination. Ultrasound findings were indicative of paralytic ileus

Results

A total of 44 dogs (23 males and 21 females) with signs of small intestinal obstruction were entered in this study. They ranged in age from 3 months to 15.5 years (mean: 4 years).

Of the 15 dogs that underwent abdominal surgery, 13 had the final diagnosis of complete or incomplete obstruction. Among the other two dogs without obstruction, one had paralytic ileus while the other had intestinal perforation.

The remaining 29 cases were treated conservatively and the diagnoses were established based upon the results of the clinical or post-mortem examination.

The final diagnoses and sonographic findings of the 44 cases are summarised in Table 1.

Ultrasound findings

The wall, luminal diameter, luminal content and peristaltic activity of the small intestines could be evaluated in all 44 dogs. The sonographic findings could be classified into the following 6 groups.

(1) Dilated intestinal loops with pendulous movement of the ingesta were seen in 8 cases (18%) and these cases were diagnosed sonographically as obstructive ileus. This sign was observed as "to-and-fro" movement of the echogenic gas bubbles and food particles in the dilated intestinal lumen together with the contraction of the intestinal wall. Six out of the 8 patients had complete intestinal obstruction. In one of the remaining two dogs, severe obstipation was diagnosed by radiography and this patient was treated conservatively. Paralytic ileus was diagnosed in the other case during the subsequent operation.

(2) In two cases (5%), the typical sonographic signs of invagination were present (i.e. multi-layered appearance of the bowel, concentric rings sign), and the diagnosis was proven by laparotomy in both dogs.

(3) Dilated intestines with varying degrees of peristaltic activity (some loops with increased, while others with decreased peristalsis) were seen in one dog (2%), which was diagnosed during laparotomy to be a case of incomplete ileus caused by an intramural neoplastic process.

(4) Sonographic signs of paralytic ileus together with abdominal fluid accumulation were found in two dogs (5%). Surgery revealed intestinal volvulus in one of them, and an intestinal perforation caused by an ingested foreign body in the other.

(5) Local or generalised paralytic ileus was found in 13 cases (30%). Among them, 10 dogs had moderate to severe enteritis, one dog had a pancreatic tumour, one had long-standing complete obstruction and one had small intestinal perforation caused by septic inflammation of the intestinal wall.

Table 1

Final diagnoses and ultrasound findings of the 44 cases examined

Final diagnosis	No. of cases	Ultrasound findings					
		"to-and-fro" movement of the ingesta	invagination or visible foreign body	non-uniform peristaltic activity	paralytic ileus and free fluid accumulation in the abdomen	paralytic signs	normal findings
Intestinal obstruction was present	13	6	2	1	2	1	1
ingested foreign bodies	8	6			1*	1	
invagination	2		2				
intestinal adhesion	1						1
intestinal volvulus	1				1		
intestinal tumour	1			1			
Intestinal obstruction was not present	31	2	–	–	–	12	17
enteritis (incl. parvoviral enteritis)	17					10	7
pancreatitis – pancreatic tumour	4**					1	3
obstipation	3	1					2
inflammation with intestinal perforation	1					1	
disorders of other organs (e.g. renal insufficiency)	6**	1					5
Total number of cases	44	8	2	1	2	13	18

*In this case, an ingested foreign body caused intestinal perforation and septic effusion; **The structural changes in the pancreas and other abdominal organs (liver, kidneys) could be readily detected by ultrasonography during the examination

(6) In 18 cases (41%) non-distended or only moderately distended bowels were found together with normal or slightly increased peristalsis during the ultrasound examination. All of the dogs but one had either mild enteritis, pancreatitis, obstipation, or organic dysfunction of other abdominal organs and were treated conservatively. The remaining dog was found to have a chronic intestinal adhesion by laparotomy.

Obstruction was correctly diagnosed by ultrasonography in 11 of the 13 dogs with mechanical ileus, and was correctly excluded in 29 of the 31 non-obstructive cases. Thus, the previously described sonographic criteria had 85% sensitivity and positive predictive value, and 94% specificity and negative predictive value. The comparison of the final diagnoses and the ultrasound results, together with the sensitivity, specificity, positive and negative predictive values of ultrasonography can be found in Table 2.

Discussion

Human studies have proven the value of ultrasonography in the diagnosis and management of different forms of ileus (mechanical and paralytic) in human beings (Truong et al., 1992; Ko et al., 1993; Ogata et al., 1994, 1996). The criteria that are used in human medicine for bowel obstruction are based on the observation of a distended bowel proximal to a non-distended one, and observation of peristaltic activity in the distended proximal bowels (Ko et al., 1993; Ogata et al., 1994, 1996). The authors of these papers also mention the "to-and-fro" movement of the ingesta inside the dilated intestinal loops proximal to the site of the obstruction (Ogata et al., 1994, 1996). Recently, duplex Doppler technique has also been proposed for observing the altered peristaltic movement of ingesta in the obstructed bowel in human cases (Gimondo et al., 1995).

Not all of these criteria can be applied to veterinary medicine, because differentiating between the different small intestinal loops is not possible (Penninck et al., 1989). Among the sonographic signs of ileus in the dog, paralytic ileus has been described (Penninck et al., 1990). Descriptions of the sonographic picture of invaginated intestines and foreign bodies can also be found in the veterinary literature (Fluckiger and Arnold, 1986; Kantrowitz et al., 1988; Watson et al., 1991; Tidwell and Penninck, 1992; Kramer and Gerwing, 1996). In one article, Tidwell and Penninck (1992) mention other sonographic signs of mechanical ileus that were seen in dogs with ingested foreign bodies (increased peristaltic activity, ascites, lymphadenopathy and pancreatitis). However, no study has evaluated the value of the observation of these signs in the diagnosis of mechanical ileus in the dog.

Table 2

Comparison of the ultrasonographic and the final diagnoses in determining whether intestinal obstruction was present. Ultrasound diagnosis was based on the observation of signs listed in Table 1

Ultrasound diagnosis	Intestinal obstruction was present	Intestinal obstruction was not present	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Obstruction	11 of 13 (TP)	2 of 31 (FP)	85%	94%	85%	94%
No signs of obstruction	2 of 13 (FN)	29 of 31 (TN)				

TP = true positive cases; FP = false positive cases; TN = true negative cases; FN = false negative cases. Sensitivity is calculated as TP/TP + FN, and specificity is calculated as TN/TN + FP. Positive predictive value is calculated as TP/TP + FP, and negative predictive value is calculated as TN/TN + FN

Two veterinary articles have described the sonographic image of gastrointestinal foreign bodies (Tidwell and Penninck, 1992; Kramer and Gerwing, 1996). During our study we did not find any ingested foreign body that transmitted the ultrasound beam. Foreign bodies have only rarely been encountered by the authors even outside this study period. This finding is in accordance with the results of Kramer and Gerwing (1996), who have seen foreign bodies only in half of their reported cases. The echogenic reflex (caused by foreign materials that reflect the ultrasound) and the acoustic shadowing (caused by reflection and/or absorption of the ultrasound beam) were seen in some of our foreign body ileus cases. According to Tidwell and Penninck (1992), the acoustic shadowing of a foreign body can be differentiated from the shadowing of intraluminal gas by the lack of internal echoes inside it. Our previous experience, however, did not prove the reliability of this sign in differentiating foreign bodies from gas accumulation inside the bowel lumen, and this fact is also mentioned by Tidwell and Penninck (1992). Thus, we did not include this sign among the sonographic criteria for diagnosing intestinal obstruction. We also did not include the measurements of the luminal diameter of the dilated intestinal loops, because we believe that these data in themselves do not help in differentiating between mechanical obstruction and paralytic ileus.

In our study we tried to establish and evaluate sonographic criteria for gastrointestinal obstruction in the dog, by using four different sonographic signs to diagnose intestinal obstruction.

(1) Our first criterion was the observation of pendulous movement of the ingesta. This can be explained by the increased peristaltic activity of the obstructed bowel that tries to move the intraluminal content of the intestine to aboral direction, but the ingesta is reflected back from the site of the obstruction. We expected this sign to be very sensitive for foreign body ileus and the results of this study have proven that our expectations were correct (6 out of 8 dogs showed this sign). Long-standing obstruction had caused paralysis in two cases, which explains why it was not possible to find this sign in those dogs. In addition, there was pendulous movement of the ingesta in two dogs without small bowel obstruction. In one of them, this movement was seen in only one intestinal loop and may be explained by the severe obstipation in the colon that could have mimicked the sign of obstruction in the ileum or distal jejunum. In the other dog, we could not find the reason for the observation of this sign, as only paralytic bowels were found during surgery. Here it is necessary to call attention to the fact that the pendulous movement of ingesta may be mistaken for the passive flowing movement of fluid inside a paralytic intestine or colon. This passive "to-and-fro" flow is caused by the animal's respiratory movements, and not by peristaltic activity, therefore a swirling movement of echogenic spots in the ingesta or intestinal wall contractions cannot be observed in those cases. Duplex Dop-

pler ultrasonography may also be useful in differentiating between the two forms of ingesta movement but it has not been applied to canine patients yet.

(2) Before this study period, we sometimes found dogs with dilated intestinal loops where both increased and decreased peristaltic activity could be observed during ultrasonography. Those dogs suffered from incomplete obstruction. That is why we included this sign among our criteria. During this study, this sign was observed in one case, and laparotomy revealed an intestinal tumour causing incomplete obstruction. Intestinal tumours can be readily detected ultrasonographically as thickening of the intestinal wall and disruption of its layered appearance (Penninck et al., 1990). We could not find wall thickening in this case, which may be explained by the gas accumulation in some of the intestinal loops that prevented visualisation of the affected part of the gastrointestinal tract.

(3) The sonographic appearance of invaginated intestinal loops has been described as intestine in intestine, hayfork sign, concentric rings sign, multilayered appearance of the bowel etc. (Fluckiger and Arnold, 1986; Kantrowitz et al., 1988; Penninck et al., 1990; Watson et al., 1991). The known high sensitivity of ultrasonography for detecting invagination has been demonstrated in two dogs during this study.

(4) Finally, we also included among our criteria the observation of paralytic intestines together with abdominal fluid accumulation. This criterion was brought from human studies where it proved to be a sensitive indicator for strangulation (Ogata et al., 1994, 1996). Of the two dogs in which we observed this sign, one had intestinal volvulus, and the other had an ingested foreign body that caused mechanical obstruction, perforation and septic effusion.

As in the case of previous human reports, the diagnostic values (sensitivity, specificity, positive and negative predictive values) of ultrasonography were high also in our study. This indicates that ultrasonography may have an important role in the diagnosis of small intestinal obstruction of the dog. By using the above-mentioned criteria it is possible to reliably diagnose or exclude intestinal obstruction in the majority of cases. The decision regarding treatment (surgery versus conservative) should be the result of both ultrasonography and the clinical examination. This approach is of special importance when paralytic ileus is found during the ultrasound examination, as this condition may also be caused by a long-standing obstruction. The comparison of ultrasonography and plain film radiography would be important for a better evaluation of the role of ultrasonography among other imaging modalities in the diagnostic process of canine ileus.

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EVALUATION OF THE NUTRITIONAL VALUE OF THE AMARANTH PLANT I. RAW AND HEAT-TREATED GRAIN TESTED IN EXPERIMENTS ON GROWING RATS

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Two experiments were carried out on rats to determine the nutritional value of raw and autoclaved amaranth (*Amaranthus retroflexus*) seeds. The test groups of the first experiment were fed only ground amaranth seed. The control group received pure maize meal. Both test groups showed higher growth rate than the control group, due to the higher protein content of amaranth. The final body weight of rats fed autoclaved amaranth seed significantly exceeded that of rats fed raw amaranth seed. The Net Protein Utilisation (NPU) index of raw and autoclaved amaranth was determined in the second experiment. The diets had 10% crude protein concentration and the only protein source was the meal of raw and autoclaved amaranth seed. It was found that the heat treatment of amaranth increased the body weight gain of rats even when the animals consumed diets containing only 10% crude protein. The feed consumption was also higher with the diet containing autoclaved seeds. Despite the finding that there was no significant difference in the NPU index, data suggest the presence of a heat-labile antinutritive compound.

Key words: Amaranth grain, rat growth test, net protein utilisation, protein quality, heat treatment, antinutritive factor

Protein-energy malnutrition is one of the major nutritional problems in the developing countries. The search for high-quality protein feeds of plant origin has led to the rediscovery of grain amaranths as potential feeds of high nutritional value. Amaranths were stale foods of the pre-Columbian civilisations of Middle and South America, but then fell into oblivion for centuries. The second discovery of *Amaranthus* species was due to their resistance to climatic environment and weather conditions, and the fact that their grain and green parts are both useful for feeding.

Taxonomically the amaranth is assigned to the division of Angiospermae, the family of Centrospermae, the class of Dicotyledonae, and the genus of *Ama-*

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ranthus. Domesticated amaranth is a broad-leafed, annual plant with a strong 0.5–2 m high stalk. It produces small (1.0–1.5 mm in diameter, 0.5 mm thick), lenticular seed which varies in colour from yellowish-white to pink, brown or even black.

It is extremely adaptable to varying climatic and agronomic needs and is tolerant of drought. The water requirement of amaranth is less than half of that of the major cereals. It produces reasonable yields even when cultivated on poor soils. Fertilisation has no effect on the grain yield but increases the greenness. The growing plant prefers warm temperature and light and it is sensitive to frost. The yield of seeds ranges from 2000 to 4000 kg/ha (lb/acre), depending on the species (Szőcs, 1995).

The cultivation of amaranth is not difficult, although it requires more human labour, attention and care than the usual crops. Weed control has to be solved without herbicides; therefore, amaranth can easily be grown organically.

The recent "career" of amaranth and the rapid spread of its cultivation are mainly due to the use of amaranth as forage in dry countries like Kazakhstan, the inner areas of China, the Ukraine and Mexico, among others. The fresh yield can reach 100 t/ha, and its quality matches that of green lucerne. The whole green plant can be used as forage or silage for cattle, sheep, swine, poultry and other domesticated animals. Amaranth can produce a considerably high amount of good-quality fresh green material without irrigation and fertilisers, even on poor soils and in a dry climate.

The approximate composition of the widely grown amaranth seeds is considered to be of high value. The dry matter content of the amaranth grain is about 90–94%. It contains about 15–18% crude protein, 6–8% ether extract, 3–5% crude fibre, 2–3% ash, and 60–65% N-free extract (Carlsson, 1979; Bressani et al., 1987; Bressani et al., 1992; Szelényi-Galántai and Zsolnai-Harczi, 1992). The protein content of the grains is higher and their amino acid composition more balanced than those of conventional cereals. The protein of the seed is rich in sulphur-containing amino acids and lysine. Satisfactory lysine and tryptophan contents, along with a reputed leucine deficiency, should make them valuable complements to leucine-rich, lysine- and tryptophan-poor maize and sorghum (Bressani et al., 1989; Vetter, 1994). The lipid concentration of amaranth grain is relatively high and its fatty acid composition is favourable. The lipid contained by the seed is rich in linoleic acid, oleic acid and palmitic acid, with a small amount of other fatty acids (Lorenz and Hwang, 1985).

When amaranth seed was fed to experimental animals, relatively high protein qualities were documented in most cases (Betschart et al., 1981; Pond et al., 1991). In some cases feed consumption, weight gain and protein efficiency ratios were lower than expected.

Problems with feed refusal toxicity have been reported when raw grain was fed (Cheeke and Bronson, 1979); however, autoclaving the grain resulted in

favourable growth and feed utilisation by chicks (Waldroup et al., 1985; Laovoravit et al., 1986). It has been suggested that a feed refusal factor exists in the grain, which is partially or completely destroyed by heat treatment.

Afolabi et al. (1981) studied *A. caudatus* grown in Africa. They found a high true protein digestibility (92%) but a surprisingly low biological value (46%) and a negative protein efficiency ratio (PER). These authors hypothesised that antinutritive factor(s), probably tannins, would have been responsible.

Koeppe et al. (1985) isolated trypsin inhibitors from seeds of *A. hypochondriacus*. The isolated inhibitor was submitted to heat resistance test. At 100 °C, during the first hour there was a relatively rapid (40%) loss of inhibitor activity, but 20% of the original trypsin inhibitor activity still remained after 7 hours of heat treatment. Small amounts of phenolic type inhibitors have been detected by HPLC (Pedersen et al., 1987*a*).

Pedersen et al. (1987*a,* 1987*b*) determined the approximate chemical composition of grains of four different *Amaranthus* species and studied the protein quality of grains in balance experiments using growing rats. They also examined the effects of three different processing technologies (popping, toasting and flaking). The grains contained small amounts of tannin (0.3%) and heat-labile protease inhibitor. The digestibility of protein was high (87%), and practically unaffected by processing. Protein digestibility of the black seeds was lower and it was also affected by processing. The nutritive value of the amaranth grain has been described by BV (biological value) and NPU (net protein utilisation) indices. The BV indices measured in several groups did not differ significantly from each other, ranging from 83 to 87%. The NPU indices were found to be about 75%. The inter-group differences in NPU indices were not significant. The protein quality of amaranth was higher than that of the other cereals.

Pant (1983) investigated the protein quality of amaranth and its supplementary effect on wheat protein. The net protein ratio (NPR) of grain amaranths (3.82) was significantly higher than that of wheat (2.68), and supplementation with amaranth protein significantly increased the NPR of wheat protein. Some earlier reports suggested that methionine is the limiting amino acid in amaranth protein. The author found that the weight gains and NPR of rats fed amaranth grain alone or supplemented with 1% DL-methionine were not significantly different from each other, implying that these amino acids were present and highly available.

The grain has been studied as a feed ingredient for poultry by various investigators (Waldroup et al., 1985; Laovoravit et al., 1986; Tillman and Waldroup, 1986, 1987, 1988*a*, 1988*b*; Acar et al., 1988). In the trial carried out by Laovoravit et al. (1986), heat-treated amaranth grain was concluded to be a satisfactory feedstuff for poultry.

Waldroup et al. (1985) carried out trials using broiler chickens. Seeds from two species of amaranth (*A. cruentus* and *A. hypochondriacus*) were incor-

porated into diets at levels of 20 and 40%, in raw and autoclaved form. The weight gain of chickens fed diets with 20% amaranth did not differ significantly from that of chickens fed the control diet, irrespective of whether the grains were raw or autoclaved. The feed conversion rate (FCR) improved at that level. The body weight gain and feed intake of chickens fed diets containing 40% amaranth were significantly reduced.

Heat treatment is necessary for partially or completely destroying the antinutritive factors that exist in the grain (Koeppe et al., 1985). Methods of heat treatment include autoclaving, popping and extruding (Pant, 1985; Imeri et al., 1987; Bressani et al., 1992). Steam extrusion of grain amaranth appears to be the most appropriate method of heat treatment (Mendoza and Bressani, 1987).

In Hungary, amaranth research and development started in 1985. Botanists, plant breeders, agronomists, food and feed experts worked for the introduction and development of this new crop. As a result, in early 1994 we obtained the first state-recognised commercial Hungarian amaranth variety, called "Edit" (Szőcs, 1995).

It is becoming more and more obvious that the global climatic changes affect our region by causing increasingly frequent and prolonged dry and hot periods in summer, posing a growing risk of drought and loss of yields. Therefore, we need here, more than ever, every possible drought-resistant crop such as amaranth. In the light of the previous and recent feeding experiments conducted in Hungary, both amaranth seed and green forage promise to become a successful new feed crop in this country already in the near future (Szelényi-Galántai and Zsolnai-Harczi, 1992; Szőcs, 1995).

The objective of the present investigation was to determine the chemical composition of home-produced amaranth and to determine the effects exerted by heat treatment on the biological value of the protein of amaranth grain.

Materials and methods

Materials

The amaranth seeds were produced by the Botanical and Ecological Institute of the Hungarian Academy of Sciences (Vácrátót). The maize starch was obtained from the Starch Factory of Ászár, the potato starch from Distilling Industries of Budapest, while the vitamin and mineral mixtures from the Agricultural Combine of Bábolna. Fat and sunflower oil used in the diets were obtained from human food stores.

The amaranth seeds used in both experiments were broken by passing them through a hammer mill using a 800 μm screen. For heat treatment, whole amaranth seeds were processed by the following methods: the grains were

soaked in water (1 part water:1 part amaranth by weight) and then autoclaved for 30 min (121 °C, 0.1 MPa). After this moist-heat treatment the amaranth grains were spread on shallow trays in a layer 1–2 cm deep and dried in a drier at 60 °C for 48 h. The dried seeds were reground before incorporation into the diet.

Diets

The ingredients and the percentage composition of the experimental diets are shown in Table 1.

Preliminary diet. This diet was fed during the week before the trial, to adapt the rats to the conditions of the experiment. It was complete in all respects, with a protein content of 15%.

Experiment 1. The test groups of the first experiment were fed only ground amaranth seed. The control rats received pure maize meal.

Experiment 2. Protein-free (reference) diet. The protein-free control diet was necessary for calculating the NPU and NPR indices.

Test diets. The diets had 10% crude protein concentration and the only protein source was the meal of raw and autoclaved amaranth seed. The total ether extract content of the diets was 15%, considering the fat content of the amaranth seed.

Experimental animals

Specific pathogen free (SPF), weanling male CFY-LATI rats, derived from the Laboratory Animal Breeding Institute (LATI) of Gödöllő, were used.

Methods

Chemical composition and amino acid analyses. Analysis for moisture, protein, ether extract, crude fibre and ash were performed according to AOAC methods (AOAC, 1990). Amino acid analyses were performed by ion-exchange column chromatography.

Experimental design. The male, weanling rats of strain WISTAR were fed the preliminary diet for 5 days to adapt the animals to the conditions of the experiment. The preliminary diet was complete in all respects, its protein content was 15%. On the fifth day the rats were allocated into groups of 6 rats each, making sure that the mean body weight per group did not vary by more than ± 1.5 g and the within-group standard deviation was minimal. The initial mean body weight was 60.84 g. Six rats per treatment were housed in wire cages. The environmentally controlled room was kept at a temperature of 28 ± 1 °C. Feed and drinking water were provided *ad libitum*. The body weight of each rat was recorded at 0, 3, 6, 8, and 10 days and the feed consumption during the 10-day period was determined.

Table 1

Composition of the experimental diets

Ingredients (%)	Preliminary diet	Experiment 1			Experiment 2		
		All-maize control diet	Raw amaranth	Heat-treated amaranth	Protein-free reference diet	Raw amaranth (10% CP in diet)	Heat-treated amaranth (10% CP in diet)
Raw amaranth			100			62.50[a]	
Heat-treated amaranth				100			58.82[a]
Extracted soybean meal	31.92						
Maize meal		100					
Cooking fat	9.25				14.25	10.69[b]	10.89[b]
Sunflower oil	0.75				0.75	0.75	0.75
Potato starch	10.00				10.00	10.00	10.00
Vitamin and mineral mixture	5.00				5.00	5.00	5.00
Glucose					15.00		
Maize starch ad 100 %	43.08				55.00	11.06	14.54

[a]amount to 10% crude protein
[b]ad 14.25% ether extract in diet

Protein quality indices. NPR (net protein ratio) was obtained by dividing the weight difference of the test and control (protein-free) groups by the protein intake of the test group. NPU was calculated by a regression equation between NPU and NPR.

Statistical calculations. Animal welfare. Statistical analyses were carried out after Pearce (1965), using the EXCEL 5 software (Microsoft, 1993). This study was approved by the Animal Use and Care Administrative Advisory Committee of the Hungarian Scientific Chamber and it complies with European Union directives regarding the use of experimental animals.

Results and discussion

Approximate chemical composition

The approximate composition of raw and heat-treated amaranth grain is shown in Table 2. The dry matter content of heat-treated (and artificially dried) amaranth grain was higher than that of the raw grain (96.00 vs. 90.90 g/100 g). Heat processing had no considerable effect on the composition of the dry matter content of amaranth grain. The dry matter of raw grain contained 17.60% crude protein, 6.27% ether extract, 4.46% crude fibre, 5.02% ash and 66.65% N-free extract. The dry matter of heat-treated amaranth grain had the following composition: 17.71% crude protein, 5.95% ether extract, 5.16% crude fibre, 5.41% crude ash and 65.77% N-free extract. Compared to common cereal grains, amaranth seed contained more protein and ether extract.

Table 2

Approximate composition of raw and heat-treated amaranth seed

		Raw amaranth	Heat-treated amaranth
Dry matter	g/100 g	90.90	96.00
Ash	g/100 g DM	5.02	5.41
Crude protein	g/100 g DM	17.60	17.71
Crude fiber	g/100 g DM	4.46	5.16
Ether extract	g/100 g DM	6.27	5.95
N-free extract	g/100 g DM	66.65	65.77

Amino acid composition

Table 3 shows the amino acid composition of raw and heat processed amaranth grain, as compared to that of some conventional cereal grains. The amino acid compositions shown in Table 3 indicate that amaranth is high in ly-

sine (6.9 g/16 g N), which lysine content is about twice as high as that of any other conventional cereal (approx. 3 g/16 g N). This result is consistent with the values reported in other studies (Bressani et al., 1989; Szelényi-Galántai and Zsolnai-Harczi, 1992).

Table 3

Amino acid composition of amaranth and some conventional cereal grain (g/16 gN)

	Raw amaranth	Heat-treated amaranth	Barley	Wheat	Maize	Soybean
ASP	9.86	8.58	6.13	5.33	6.89	12.00
GLU	24.49	24.40	23.87	27.48	18.78	18.79
SER	7.18	6.66	4.62	5.05	5.11	5.59
HYS	3.46	3.60	2.18	2.24	2.89	2.49
THR	3.97	3.98	3.53	3.08	3.67	4.19
GLY	6.78	5.75	4.03	4.02	4.00	4.19
ARG	10.44	9.88	5.21	4.77	4.89	7.21
TYR	3.75	3.47	3.36	3.08	4.00	3.51
ALA	5.16	4.84	4.20	3.83	7.33	4.30
TRP	0.35	0.39	1.26	1.12	0.89	1.29
MET	1.43	1.23	1.68	1.68	2.22	1.40
VAL	3.29	3.55	5.46	4.67	5.11	4.99
PHE	3.76	4.36	5.21	4.49	4.78	4.90
ILE	2.79	3.13	3.78	3.83	3.78	4.90
LEU	5.54	5.65	6.97	6.82	12.00	7.59
LYS	6.88	6.11	3.61	2.90	3.00	6.41

Rat experiments

Changes in the body weight of rats as a function of elapsed time are shown in Figs 1 and 2. The feed intake and protein utilisation indices are summarised in Table 4 and Figs 3 and 4.

Experiment 1. Figure 1 shows that the test groups fed only raw and heat-treated amaranth grain had a higher growth rate than the control group fed only maize meal. This difference was due to the higher protein content of amaranth. The average final body weight of the control group was 68.53 g, while that of the test groups fed raw and heat-treated amaranth was 73.15 g and 85.80 g, respectively. At the end of the trial, the body weight of rats fed autoclaved amaranth proved to be significantly higher than that of rats fed raw amaranth.

Data of Table 4 and Fig. 3 show that the test animals prefer the autoclaved amaranth to raw. The average daily feed intake of rats fed heat-treated seeds was higher than that of rats receiving raw amaranth (6.83 g vs. 7.56 g).

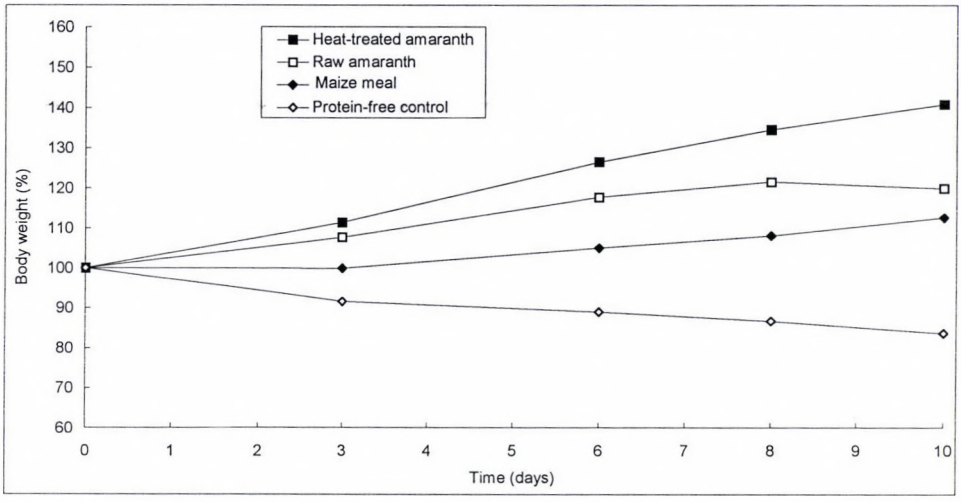

Fig. 1. Body weight curves of growing rats fed maize diet and amaranth seed diet

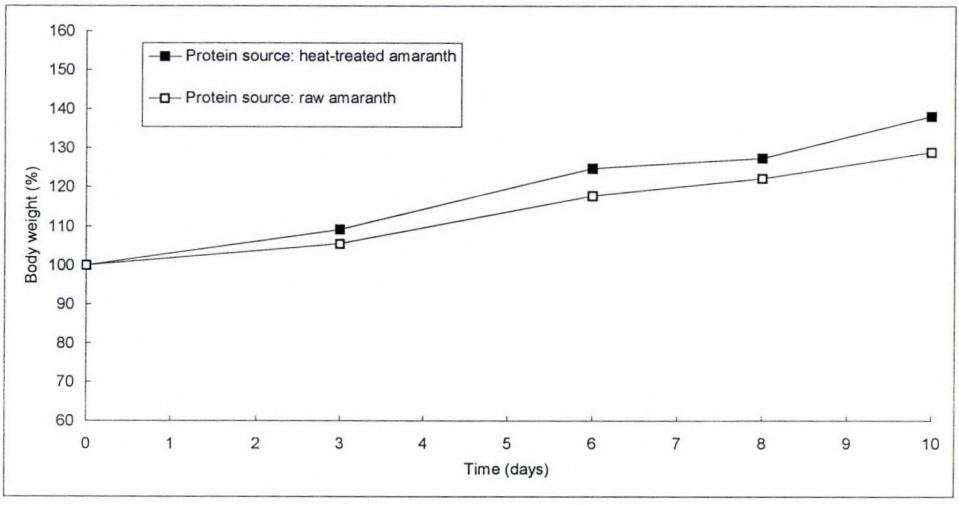

Fig. 2. Body weight curves of growing rats fed diets containing 10% protein

Knowing the protein level of diets, the feed intakes as well as the initial and final body weights, the protein utilisation indices were calculated. The NPU (Net Protein Utilisation) index of autoclaved amaranth was higher than that of the raw grain (51.2% vs. 39.0%). Heat treatment had a favourable effect on protein utilisation. The higher NPU value of maize meal (55.1%) probably can be attributed to its lower protein level. Protein utilisation is in inverse ratio to the protein level of diets (Hegedűs et al., 1981).

Table 4

Growth performance, feed consumption and protein utilisation of rats

	Diet	Final body weight (W)	Protein level in diet	Feed consumption	Protein consumption (I)	NPR[1]	NPU[2]
		(g)	(%)	(g/rat/10 days)	(g/rat/10 day)	(g/g)	(%)
Experiment 1	Maize meal control	68.53 ± 3.80	8.43	73.10	6.16	2.94	55.10
	Raw amaranth	73.15 ± 3.48	16.02	68.27	10.94	2.08	39.00
	Heat-treated amaranth	85.80 ± 5.40	17.15	75.62	12.97	2.73	51.20
Experiment 2	Protein-free reference diet	50.42 ± 3.33 (W_k)	0.00	43.35	0.00	–	–
	Raw amaranth (10% CP)	78.53 ± 4.43	10.00	70.98	7.10	3.96	74.20
	Heat-treated amaranth (10% CP)	84.08 ± 4.08	10.00	83.70	8.37	4.02	75.40

[1] $NPR = \frac{W - Wk}{I}$

[2] NPU = 3 NPR (Hegedűs et al., 1981)

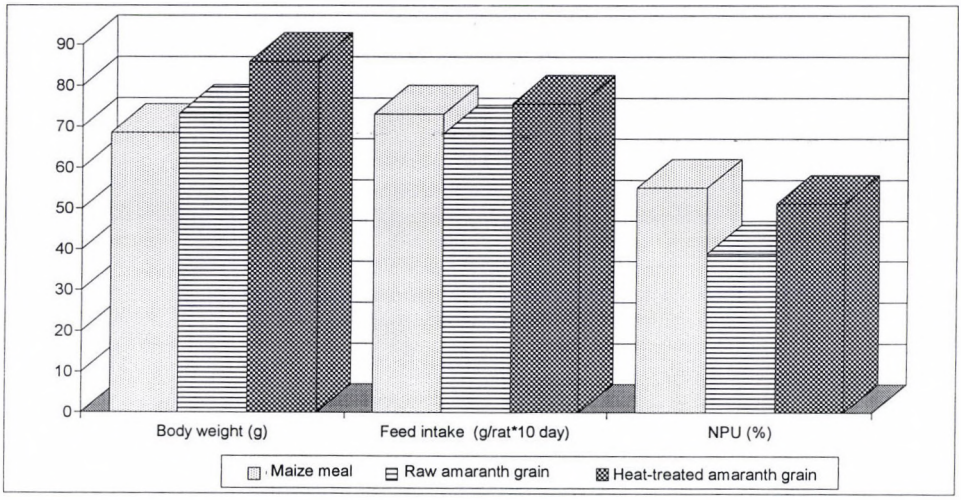

Fig. 3. Effect of heat processing on final body weight, feed consumption and NPU index (Experiment 1)

Experiment 2. The raw and the heat-treated amaranth was fed in diets of 10% crude protein concentration. (The qualities of different protein sources are commonly determined in diets containing 10% crude protein, because protein utilisation has its maximum at this level.) The single protein source of these diets was amaranth meal. The composition of the diets is given in Table 1.

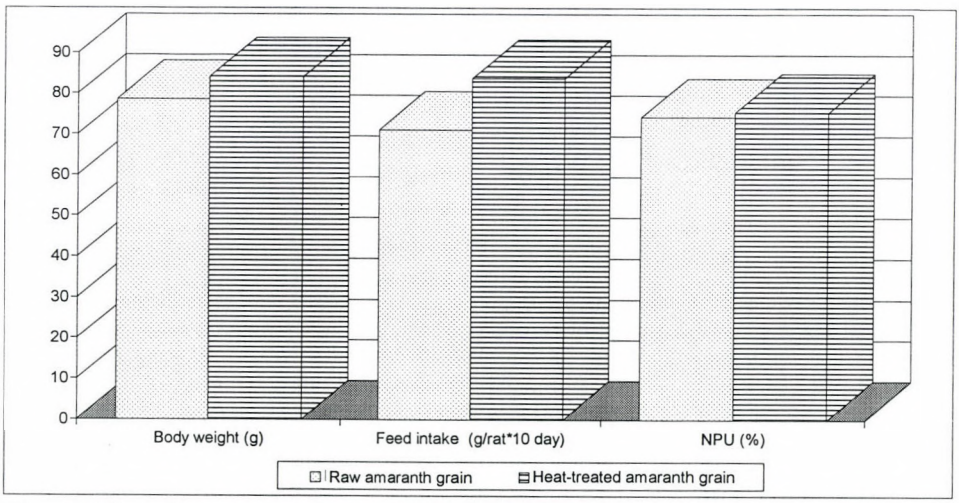

Fig. 4. Effect of heat processing on final body weight, feed consumption and NPU index (Experiment 2)

Figure 2 shows that the heat treatment of amaranth grain favourably influenced the body weight gain even at a 10% crude protein level. Nevertheless, the difference between the two test groups in final body weight was not significant ($p > 0.05$). The average final body weight of the group fed autoclaved amaranth was 84.08 g while that of the group fed raw amaranth was 78.53 g (Table 4). The experimental animals preferred the heat-treated seed. The average daily feed intake of the group fed autoclaved amaranth was 8.37 g while that of the group consuming raw grain was 7.1 g.

The NPU index of autoclaved amaranth slightly exceeded that of the raw grain (75.4% vs. 74.2%; Fig. 4). This implies that the raw seed contains some antinutritive factors which can be neutralised by the heat treatment applied.

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ASSESSMENT OF NUTRITIONAL ADEQUACY OF THE PROTEIN IN DOG FOODS BY TRIALS ON GROWING RATS

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In order to offer methods for assessing the protein quality of dog foods, eight commercial dry-type complete dog foods were analysed by *in vitro* chemical methods (crude protein, amino acid composition, *in vitro* pepsin digestibility) and rat growth trial (weight gain = WG, feed efficiency = FE, protein efficiency ratio = PER, net protein ratio = NPR, and net protein utilisation = NPU). Three individual samples were taken from each dog food and their homogenous mixture was used as an ingredient for the test diets fed to six rats per diet. All dog food samples were used as a single protein source in isonitrogenous (10% crude protein) and isoenergetic (ME = 14.9 MJ/kg) complete diets. Rat weight gains showed significant differences among most of the test diets as a consequence of the differences in protein quality of the dog food samples. The protein quality indices of the dog food products showed significant variations. The measured ranges of protein quality indices of the samples were as follows: WG = –5.4–21.4 g; FE = 0–0.25 g/g; PER = 0–2.54 g/g; NPR = 1.06–3.52 g/g; NPU = 19.93–65.93%. The limiting amino acid, the sum of essential amino acids and the chemical scores were calculated from gross amino acid composition. Tryptophan was the first limiting amino acids in all samples but one. Methionine was found to be the second limiting amino acid. The crude protein content of the samples showed no correlation with indices of protein quality, indicating that the declaration of crude protein is not a sufficient information to judge the protein quality of dog foods. The declaration of NPR, or NPU indices may be recommended for labelling the protein quality of premium-type dog foods.

Keywords: Dog foods, assessment of protein quality, methodological approach

Commercial dry dog foods commonly contain animal by-products, cereal grains, soybean products, by-products of the food industry, and vitamin and mineral supplements. Dry-type dog foods are often marketed as complete and balanced feeds, meeting all nutritional needs of the dog when fed alone (Case et

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al., 1995). However, the nutritive value of dog foods is often variable as a consequence of the variable chemical composition and nutrient content of the by-products used during the production (Bokori and Hegedűs, 1987). Although it is often claimed that premium-type dog food products contain proteins of high quality, the numerical expression of the protein quality on the labelling is regularly missing, or neglected.

Rats are widely used as test animals for assessing the protein quality of feedstuffs for animals having similar essential amino acid requirements (Pellett and Young, 1980). Dogs require the same essential amino acids as rats, and the pattern of amino acids of the protein ideal for both species is similar as well (NRC, 1985). The rat has also been shown to be a useful model for examining the quality of proteins fed to dogs (Burns et al., 1982).

The purpose of this research was to investigate appropriate methods for screening the protein quality of various dog foods, which may then be used to numerically declare their protein quality.

Materials and methods

Dry-type dog food samples were obtained at random from commercial sources: "Frolic" (produced by Effem Ltd., Germany), "Bento" (produced by Versele-Laga, Belgium), "Pedigree Pal" (produced by Master Foods, Poland), "Feed Full" (produced by EXTAK Ltd., Hungary), "ZoBioSi" (produced by Biogazda Ltd., Hungary), "Falhat" (produced by Bábolna Ltd., Hungary), "Hamm" (produced by Pamax Ltd., Hungary), "Kedvenc" (produced by ATEV Co. Ltd., Hungary). In the following the various brands of dog food will randomly be represented by A, B, C, D, E, F, G and H, because the main objective of the study is to evaluate the methods of assessment of nutritional adequacy of protein in various products rather than to describe a specific dog food product.

Three packages of 5 kg weight were taken from each dog food product and then mixed. The homogenous mixtures were used as ingredients for the test diets.

The protein quality of the dog foods was tested in a 10-day growth assay with weanling male rats (WISTAR, SPF, LATI, Gödöllő, Hungary) using the carcass method as described originally by Pellett (1973) with the following modifications: rats were housed in groups of six per wire cage, feed and water were provided *ad libitum*. The temperature of the animal room was 28 °C and the humidity was maintained between 60–80%. Animals were assigned so that all treatment groups had the same initial mean body weight. The composition of the test diet was: cooking fat (pork) 14.25% (w/w), sunflower oil 0.75% (w/w), cellulose powder 5.00% (w/w), vitamin and mineral premix 1.00% (w/w), dog food sample to give 10.0 g/100g crude protein in the test diet and maize starch

ad 100% (w/w). The vitamin and mineral premix supplied the following vitamins and minerals in the diet: vitamin A: 6000 IU/kg, vitamin D: 1000 IU/kg, vitamin E: 50 mg/kg, thiamine: 4 mg/kg, riboflavin: 3 mg/kg, niacin: 20 mg/kg, Ca-pantothenate: 8 mg/kg, pyridoxine: 6 mg/kg, vitamin B_{12}: 50 μg/kg, Ca: 5 g/kg, P: 4 g/kg, Zn: 12 mg/kg, Mn: 50 mg/kg, Fe: 40 mg/kg, I: 0.15 mg/kg, Se: 0.1 mg/kg. A protein-free diet was used for considering endogenous protein losses. The composition of the protein free diet corresponded to that of the test diet but maize starch was used instead of the dog food sample.

The criteria indicating the performance of the rats fed the test diets were as follow: weight gain (WG), feed efficiency (FE), protein efficiency ratio (PER), net protein ratio (NPR), and net protein utilisation (NPU). FE was defined as weight gain (g) at the end of feeding period related to one gram of feed consumed. PER was calculated as weight gain per weight of protein intake (g/g). NPR is a modified index of PER and is calculated as follows: weight gain of a test animal, plus weight loss of the control animal fed protein-free diet, per gram of protein consumed by the test animal (g/g). NPU (%) was calculated from the linear regression equation existing between NPR and NPU (Hegedűs, 1984). NPU expresses the retained part of the consumed protein in percentage.

Proximate chemical analysis was performed and pepsin-soluble N content of the samples determined according to the corresponding Hungarian standard MSZ 6830. Amino acid analyses were performed by ion-exchange chromatography, except for tryptophan, at the Central Laboratory of the Technical University of Budapest. Tryptophan was determined according to the method of Matheson (1974).

The limiting essential amino acid, the sum of essential amino acids and the amino acid scores were calculated from the gross amino acid content of the samples using the amino acid pattern of the ideal protein for rats (NRC, 1978) as a reference amino acid composition. Amino acid scores express the quantity of the amino acid in question relative to its requirement. The amino acid score of the limiting essential amino acid has been called chemical score (CS).

MS Excel 5.0 software (Microsoft, 1995) has been used for statistical processing of the data for significant differences between the mean values.

Results and discussion

Chemical composition and amino acid content

The results of the proximate analysis of the samples and amino acid composition of the protein are summarised in Tables 1 and 2. The range of *crude protein* content of the samples was 21.8–38.8 g/100 g. Numerous factors may

modify the percentage of protein required in the diet, such as digestibility, amino acid composition of the protein, availability of amino acids, caloric density of the diet, and physiological state of the dog. It is therefore difficult to suggest a clear-cut crude protein level in dog foods. The Waltham Centre for Pet Nutrition (WCPN) recommends 9.6 g protein/MJ metabolisable energy as the minimum maintenance requirement in foods for adult dogs (Burger, 1993), which corresponds to approximately 14–15 g protein/100 g food depending on the food's particular metabolisable energy (ME) content. However, in commercial complete dry-type dog foods for adult dogs with moderate activity a minimum of 18 g protein/100 g food is recommended to ensure that minimum protein requirements are more than adequately met (AAFCO, 1993).

Table 1

Proximate chemical composition and pepsin-soluble N content of dog food samples

Dog food*	A	B	C	D	E	F	G	H
Moisture (g/100)	7.3	4.9	6.0	5.3	6.0	5.9	10.4	6.5
Crude protein (g/100 g)	24.6	38.8	33.8	22.9	26.3	32.5	21.8	22.2
Ether extract (g/100)	11.1	6.6	9.4	6.0	8.9	6.4	15.2	5.6
Crude ash (g/100 g)	7.1	19.6	9.7	9.0	16.7	7.1	7.7	8.6
Crude fibre (g/100 g)	0.4	2.9	1.2	2.1	4.3	1.5	2.5	0.8
N-free extractives (g/100 g)	49.5	27.2	39.9	54.7	37.8	46.6	42.4	56.3
Pepsin-soluble N (g/100 g)	89.5	81.0	85.1	90.2	80.4	90.1	84.5	98.0

*The letters represent different brands of dog foods

The high *ash content* in two samples (19.6 g/100 g in "B", as well as 16.7 g/100 g in "E") indicates a possible high bone ratio among raw materials used in these diets. The consequence of the high bone content may be the high collagen ratio within the crude protein originating from bone protein. Collagen is low in essential amino acids, mainly in lysine, tryptophan and cystine, therefore the high ash in a dog food sample may reflect a lower quality of the protein as well. Thus, the determination of the ash content of a sample may give some information regarding its protein quality (Hegedűs and Bokori, 1983). The sum of essential amino acids was found to be the lowest in these two samples (Table 2).

The *ether extract* (crude fat) content ranged from 5.6 to 15.2 g/100 g in the samples. The higher fat levels may improve the palatability of the dry-type food, and supply adequate linoleic acid, improving hair coat sheen (Fekete and Vörös, 1994).

Table 2

Amino acid composition of the protein (g/16 gN) and limiting essential amino acid of dog food samples

Dog food*	A	B	C	D	E	F	G	H
Lysine	5.7	3.6	7.4	5.4	4.3	5.3	6.4	4.3
Methionine	1.3	1.0	0.7	1.1	0.8	1.1	1.5	2.9
Cysteine	0.8	0.5	0.5	0.6	1.2	1.0	1.0	0.6
Tryptophan	0.1	0.3	0.2	0.1	0.1	0.2	0.4	0.7
Threonine	3.3	2.5	3.8	3.2	3.2	3.1	3.7	3.3
Phenylalanine	4.2	2.3	4.8	3.9	3.6	4.0	2.9	2.8
Tyrosine	2.7	2.0	2.1	2.6	2.1	2.8	3.4	2.7
Leucine	9.3	5.8	9.3	7.5	6.2	7.8	7.4	5.8
Isoleucine	3.1	3.0	2.2	3.2	3.2	3.2	3.9	3.2
Valine	6.0	3.4	6.4	4.6	4.5	3.9	3.5	3.3
Histidine	4.5	2.1	4.5	3.1	1.8	2.2	5.9	1.7
Arginine	5.6	3.9	5.9	5.9	6.6	5.6	6.6	4.6
Glycine	5.9	3.2	7.6	8.2	9.1	5.2	5.6	3.0
Alanine	5.8	3.2	6.3	5.9	4.9	5.0	4.4	5.4
Serine	5.0	4.3	5.0	4.6	5.0	3.7	4.6	2.8
Proline	6.4	4.2	7.4	6.3	10.4	7.3	7.3	2.6
Glutamic acid	12.9	10.1	12.1	14.2	17.1	14.5	17.0	9.2
Aspartic acid	9.3	6.0	9.5	7.9	6.5	6.7	10.6	8.6
SAA**	91.9	61.4	95.7	88.3	90.6	82.6	96.1	67.5
SEAA***	36.5	24.4	37.4	32.2	29.2	32.4	34.1	29.6
Limiting AA	TRP	TRP	TRP	TRP	TRP	TRP	TRP	PHE

*The letters represent different brands of dog foods; **Sum of amino acids; ***Sum of essential amino acids (LYS, MET+CYS, TRP, THR+PHE, TYR, LEU, ILE, VAL)

In vitro pepsin digestibility of the protein (pepsin-soluble N content) exceeded 80%, predicting acceptable *in vivo* digestibility. The pepsin-soluble N content of the samples, however, showed no correlation with their crude protein levels (Table 5).

The *limiting essential amino acid* in the protein of the samples was in most cases tryptophan; however, methionine may also play a limiting role (Table 3). Chemical score values showed only limited correlation with the other indices of protein quality (Table 5).

Carnivorous animals may have an essential arginine requirement to prevent ammonia intoxication in blood (Burns et al., 1981). Arginine scores of the protein in the samples have indicated above-requirement arginine levels in most cases (Table 3).

Table 3

Essential amino acid scores* of dog foods

Dog food**	A	B	C	D	E	F	G	H
Lysine	116.60	73.90	151.90	110.70	87.70	108.20	130.90	86.70
Methionine	34.30	26.20	18.10	28.40	22.50	29.80	41.00	78.80
Tryptophan	6.10	20.60	12.20	9.70	9.00	11.40	28.80	49.10
Threonine	74.20	56.30	84.90	70.70	70.10	68.40	82.30	74.30
Phenylalanine	60.70	32.80	69.60	56.90	52.60	57.50	42.30	41.20
Leucine	168.10	104.50	169.00	135.60	112.40	141.80	135.20	105.20
Isoleucine	90.50	89.00	63.40	95.00	94.40	93.10	114.90	93.40
Valine	161.70	91.70	174.10	124.50	121.50	106.20	93.60	89.30
Histidine***	263.30	122.10	265.90	179.20	105.10	131.20	345.40	101.40
Arginine***	116.10	81.80	122.70	122.10	138.20	116.10	138.10	95.70

*Concentration of the amino acid (g/16 gN) expressed in percentage of the requirement (NRC, 1985); **The letters represent different brands of dog foods; ***Histidine and arginine may be essential for young dogs

Utilisable protein content

The performance of the rats fed complete test diets containing dog food samples as a sole protein source to give 10% crude protein level is summarised in Table 4. To calculate indices of protein quality, weight gain and crude protein intake were measured. The final body weights and standardised indices, such as PER, NPR and NPU showed substantial differences among the samples. This can be explained by the possible differences in the source, ratio, and quality of the raw materials used in the samples. The variations in the feed intake might have played a minor role in the variations in PER, NPR or NPU values, because these indices are referred to protein intake to exclude the effect of differences in voluntary feed consumption. The palatability of a food is not necessarily similar to dogs and rats, in spite of this the ranking of different protein sources was shown to be similar (Burns et al., 1982).

The crude protein content of the samples showed poor correlation with indices of protein quality, suggesting that the declaration of crude protein alone may not provide sufficient information to allow evaluation of the protein quality of dog foods. There was a close correlation between weight gain (WG) and feed intake ($r = 0.99$, $p < 0.05$). The biological indices (PER, NPR) correlated well with each other ($r = 0.95$, $p < 0.05$), however there was much weaker correlation between biological indices (PER, NPR) and chemical score ($r = 0.51$, as well as 0.46). The sum of essential amino acids in the protein showed no correlation with crude protein, PER and NPR.

Table 4

Mean performance data of the rats at the end of the 10-day feeding period

Type of diets	Protein-free control	A	B	C	D	E	F	G	H
Number of rats	6	6	6	6	6	6	6	6	6
Starting weight (g)	48.70	48.57	48.53	48.52	48.47	48.50	48.43	48.42	48.42
Finishing weight (g)[1]	40.25	52.70	50.08	43.12	48.70	47.65	63.13	69.82	59.27
SD (± g)	3.22	5.80	3.05	3.88	4.86	4.29	8.46	8.25	7.70
Feed intake (g)	30.63	48.88	46.32	27.10	44.75	41.87	67.88	84.08	63.13
Protein intake (g)	0.00	4.89	4.63	2.71	4.48	4.18	6.77	8.41	6.32
WG (g)	–	4.13	1.55	–5.40	0.23	–0.85	14.70	21.40	10.85
FE (g/g)	–	0.08	0.03	0.00	0.01	0.00	0.22	0.25	0.17
PER (g/)	–	0.84	0.33	0.00	0.05	0.00	2.17	2.54	1.72
NPR (g/)	–	2.55	2.12	1.06	1.89	1.77	3.38	3.52	3.01
NPU (%)	–	47.74	39.81	19.93	35.37	33.19	63.37	65.93	56.43

WG (weight gain) = finishing weight–starting weight
FE (feed efficiency) = weight gain per g of food intake
PER (protein efficiency ratio) = weight gain per g of protein intake
NPR (net protein ratio) = weight gain relative to a protein-free control group per protein intake
NPU (net protein utilisation) = retained protein in percentage of the protein intake
SD = standard deviation of the mean finishing weight
[1]Significant ($p < 0.05$) differences: A–C, A–G, B–C, B–F, B–H, C–F, C–G, C–H, D–F, D–G, D–H, E–F, E–G, E–H

Table 5

Correlation among the indices used for monitoring protein quality

	CP	SEAA	CS	PSN	FI	WG	FE	PER
SEAA	0.32							
CS	0.29	–0.32						
PSN	–0.48	0.21	0.50					
FI	–0.42	–0.06	0.50	0.29				
WG	–0.38	0.02	0.49	0.31	0.99^{+}			
FE	–0.31	0.12	0.51	0.41	0.94^{+}	0.98^{+}		
PER	–0.31	0.11	0.51	0.40	0.99^{+}	0.98^{+}	0.99^{+}	
NPR (or NPU*)	–0.36	–0.06	0.46	0.40	0.95^{+}	0.97^{+}	0.95^{+}	0.95^{+}

CP = crude protein; SEAA = sum of essential amino acids; CS = chemical score; PSN = pepsin-soluble N; FI = feed intake; WG = weight gain; FE = feed efficiency; PER = protein efficiency ratio; NPR = net protein ratio; NPU = net protein utilisation

*NPU has been calculated from NPR

$^{+}$significantly ($p < 0.05$) deviates from zero

FE and PER did not prove to be sensitive indices of protein quality in the tested dog food samples. The use of the modified version of PER, i.e. NPR, was regarded to be more practical, because of the use of a control group fed a protein-free diet. Thus NPR allows differentiation between lower quality samples, which do not support weight gain in rats, and can be easily calculated from the final body weights and nitrogen consumption. If the NPR method is used, the rats need not be killed at the end of the feeding period and can be used for other purposes.

To express the protein quality of a dog food by a more commonly used term, such as the retained part of the consumed protein, NPR can be converted to NPU, utilising the significant linear regression between them.

Methods applicable for regulatory purposes

For routine quality control purposes, the examination of a dog food should begin with proximate chemical analysis of the sample. To assess protein quality, the amino acid content of the protein (g/16 g N) and the pepsin-soluble nitrogen (*in vitro* pepsin digestibility) must also be determined. From the amino acid composition of the protein the limiting amino acid and amino acid scores can be determined. However, biological tests may often reveal a less favourable picture of the protein quality of dog foods, than indicated by amino acid scores. This may be explained by a possible lower digestibility of the protein and/or by reduced availability of amino acids in heat-processed ingredients used for the pro-

duction of the food. The epsilon-amino group of lysine has been shown to be susceptible to the Maillard-type reaction with reducing sugars as a consequence of the heat generated during processing, resulting in a lower availability of lysine (Hegedűs, 1990).

In vivo protein quality evaluation can be relatively easily carried out by measuring NPR, or NPU indices with growing rats, using a direct carcass method, instead of the more laborious balance experiments (Neergaard, 1981). The additional advantage of the growth method is that it helps to reveal the possible presence of growth-depressing antinutritive or toxic factors as well.

The applicability of rat NPU values for ranking dog foods may be acceptable because of the similar essential amino acid requirements of the two species (NRC, 1978; NRC, 1985). The absolute values of protein retention are of less interest from the legislative point of view, because of the many factors influencing them (Hegedűs, 1992). The concept of using rats for ranking protein sources for monogastric animals or humans, however, is not new (e.g.: Eggum, 1973; Sammons and Hegsted, 1977; Burns et al., 1982) and is widely accepted.

Based on the findings of this study, the authors conclude that the determination of NPR or NPU indices in growing rats by the direct carcass method is an acceptable and practical method for evaluating the protein quality of commercial dog foods for regulatory or labelling purposes to differentiate between high quality "premium" and lower quality "popular" type pet foods having the same declared nutrient profile (proximate analytical values).

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ENDOCRINE AND REPRODUCTIVE CONSEQUENCES OF CERTAIN ENDOTOXIN-MEDIATED DISEASES IN FARM MAMMALS: A REVIEW

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After giving an overview of the general pathology of endotoxin-mediated diseases, the authors summarise the endotoxin-induced endocrine changes and their clinical consequences, with particular regard to reproduction. The consequences of temporary activation of the cyclooxygenase-2 and lipoxygenase enzyme systems resulting in elevated release of various prostanoids are discussed in cyclic and pregnant ruminants, sows and mares. The clinical failures attributable to increased glucocorticoid secretion as well as the endotoxin-induced changes in thyroid function and in peripheral level of some other hormones (prolactin, growth hormone and insulin-like growth factor 1) are also reviewed.

Key words: Endotoxin, reproduction, mastitis, prostaglandins, cortisol, tumor necrosis factor, thyroid hormones, insulin-like growth factor 1

Abbreviation key: ACTH = adrenocorticotropic hormone; CL = corpus luteum; COX-2 = cyclooxygenase-2; CRH = corticotropin releasing hormone; C20:4 = arachidonic acid; GH = growth hormone; IGF-1 = insulin-like growth factor-1; LH = luteinising hormone; LPS = lipopolysaccharide; $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$; PGFM = 15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$; PGI_2 = prostaglandin I_2; 6-keto-$PGF_{2\alpha}$ = 6-keto-prostaglandin $F_{2\alpha}$; PRL = prolactin; SCC = somatic cell count; TxA_2 = thromboxane A_2; TxB_2 = thromboxane B_2; TNF_α = α-tumour necrosis factor; TRH = thyrotropin releasing hormone; TSH = thyrotropic hormone; T_4 = thyroxine

Pathology of endotoxins

The medical interest in endotoxins is as old as our century. The term *endotoxin* (synonyms: O, or somatic antigen) refers to the part of the cell wall of

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Gram-negative bacteria which is composed of polysaccharides, phospholipids as well as of small amounts of protein, collectively called lipopolysaccharides (LPS). The endotoxin molecule consists of 3 main subunits: (a) the O-polysaccharide, imparting serospecificity to Gram-negative bacteria, (b) the lipid moiety, which is considered to be the toxic component of the cell wall, and (c) the R core, consisting of hexoses, hexamines and heptose, which act as bridges between the other two components (Fig. 1) (Westphal, 1975; Sandholm and Pyörala, 1995). According to the similarity of cell wall structure in various Gram-negative bacteria, LPS preparations isolated from *Escherichia coli, Salmonella typhimurium, Pasteurella haemolytica* and *Enterobacter agglomerans* strains are equally used in experimental models to study the pathological consequences of endotoxins (Anderson et al., 1986; Peter et al., 1987; Daels et al., 1987; Lohuis, 1989; Cort et al., 1990; Aiumlamai, 1991; Daels et al., 1991*a*, 1991*b*; Kindahl et al., 1991; Cullor, 1992; Jarlov et al., 1992; Shuster and Harmon, 1992; Sordillo and Peel, 1992; Elsasser et al., 1995; Holst and Kindahl, 1995; Horadagoda et al., 1996).

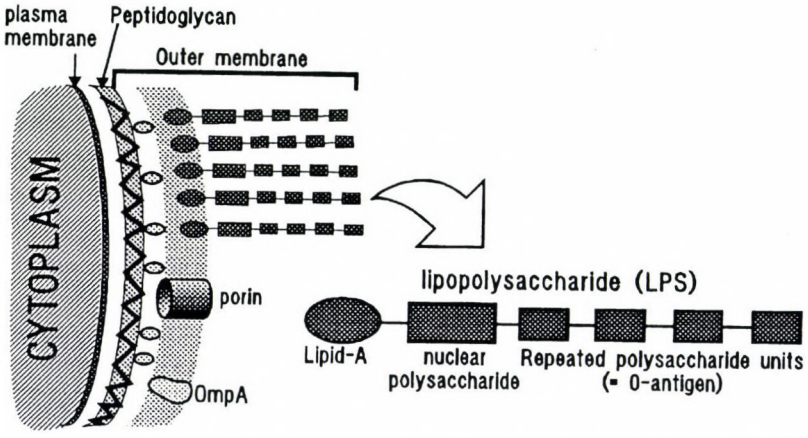

Fig. 1. The cell wall structure of Gram-negative bacteria (after Sandholm and Pyörala, 1995)

Both laboratory and farm animals can be exposed to LPS from exogenous and endogenous sources: Endotoxins can be (a) exogenously *injected* with contaminated drugs or in experimental situation, (b) *liberated* in various disease situations (mastitis, laminitis, or sepsis caused by Gram-negative pathogens), and (c) *absorbed* from the postpartum uterus or — under certain circumstances — from the rumen and/or gastrointestinal tract. Endotoxins are also present in the environment, stemming from the growth of Gram-negative bacteria on plants

and other sources of animal feed. Therefore, animals can be exposed to endotoxins not only from infection by Gram-negative bacteria, but also — at least in pathological cases — by resorption from endotoxin-contaminated fodder, and/or due to the Gram-negative bacteria residing in the gastrointestinal tract. In ruminants the rumen fluid contains large amounts of endotoxins which increase when animals are fed on concentrates instead of hay. Increased LPS absorption from the forestomachs has been demonstrated in complex disease syndromes such as ruminal acidosis, ruminal stasis and parturient paresis in the cow. In experimental models the standard endotoxin-producing (usually *Escherichia coli*) cultures, or LPS-containing preparations may be administered intravenously (as a single bolus injection, or as a some hours long, slow infusion), intramuscularly (mostly with contaminated biological preparations), intraperitoneally, and by the intrauterine, intramammary and oral route (Giri et al., 1984; Giri et al., 1990; Anderson et al., 1986; Daels et al., 1987; Peter et al., 1987; Lohuis, 1989; Cort et al., 1990; Gilbert et al., 1990; Peter et al., 1990; Yagoda et al., 1990; Aiumlamai, 1991; Boosman et al., 1991; Daels et al., 1991*a*, 1991*b*; Kindahl et al., 1991; Jarlov et al., 1992; Shuster and Harmon, 1992; Sordillo and Peel, 1992; Andersen et al., 1994*a*, 1994*b*; Kooij et al., 1994; Elsasser et al., 1995; Holst and Kindahl, 1995; Sandholm and Pyörala, 1995; Kindahl et al., 1996). Of them, the oral/gastrointestinal way of LPS loading seems to be the most problematic: Even if after oral LPS administration slight to severe clinical symptoms and also biochemical consequences of endotoxicosis have been reported to occur in both monogastric animals (pig) and ruminants (goat) (Cort et al., 1990; Holst and Kindahl, 1995), probably the portal vein endotoxaemia (without bacteraemia) of intestinal origin must be regarded as a physiological state. Endotoxins absorbed from the gastrointestinal tract must be passed to the liver where they may be removed, mainly by the Kupffer's cells (Fox et al., 1990). Bile acids are known to have decisive importance in the detoxification of endotoxins in the gastrointestinal tract (Kocsár et al., 1960; Bertók, 1983). The clearance capacity of a normal liver has been shown to exceed the endotoxin amounts normally present in the portal blood in rats (Yamaguchi et al., 1982). The endotoxin load is known to increase in different gastrointestinal disorders and thereby overload the hepatic detoxifying capacity, as in dogs with haemorrhagic enteritis (Wessels et al., 1987). Malnutrition is another condition that may lead to endotoxaemia of gastrointestinal origin: it has been shown that protein malnutrition with LPS containing nutrients may destroy the intestinal mucosal barrier (Deitch et al., 1987; Klein et al., 1988). In ruminants also the rumen can be the source of endotoxin-overload in certain cases (ruminal stasis induced by hypocalcaemia or certain drugs; ruminal acidosis) (Andersen et al., 1990; Aiumlamai, 1991; Andersen et al., 1994*b*). The impairment of liver function due to cirrhosis, hepatitis or hepatic lipidosis may also result in the permeation of endotoxins of gastrointestinal

origin to the systemic circulation (Ravin et al., 1960). The clearance ability of the liver is therefore an important factor in the prevention of the spill-over of endotoxins, which has been confirmed also in ruminants (Andersen et al., 1996).

Although limited data are available in the literature, it is generally accepted that the absorbed or experimentally administered endotoxin rapidly disappears from the circulation. Clinically healthy cows cleared endotoxin from the peripheral plasma within 30 min (Andersen et al., 1996), which may explain the limited success or failure of attempts to detect endotoxin in bovine systemic blood in Gram-negative mastitis or in ruminal disorders (Aiumlamai, 1991; Katholm and Andersen, 1992; Andersen et al., 1994*b*). According to this phenomenon, indirect haematological and biochemical consequences rather than the direct determination of LPS are used to confirm the diagnosis of endotoxicosis even under experimental conditions (Lohuis, 1989; Aiumlamai, 1991; Sandholm and Pyörala, 1995). Due to the effect of these nonsteroidal anti-inflammatory substances on liver metabolism, the plasma disappearance time of experimentally injected endotoxin was 2–3 times longer in cows pretreated with flunixin meglumine, and 6–12 times longer in cows pretreated with phenylbutazone than in their healthy counterparts (Andersen et al., 1996). An even more marked effect may be attributed to the spontaneously developed peripartal hepatic lipidosis: none of the 4 cows treated with 25 μg standard (*Escherichia coli* 055:B5) endotoxin were able to clear the injected LPS dose within the 360-min long sampling period and one of them died before the end of the study. These results support the theory that *cows suffering from hepatic lipidosis are more susceptible to endotoxins* originating from the gastrointestinal tract or from organs (udder, uterus) infected with Gram-negative bacteria than healthy cows. The often stated lack of therapeutic success in the cow suffering from fatty liver may be ascribed (at least partly) to these findings, especially when drugs depending on liver metabolism are used (Andersen et al., 1996). The phenomenon may have clinical importance in dairy cows affected by Gram-negative mastitis in the early postpartum period.

The pathophysiological consequences of endotoxin exposure have an obvious dose-dependent character, being more severe if (a) the *endotoxin neutralising capacity* of the *organ* (*rumen* in case of acidosis; *udder* in the colostral period) or of the *whole organism* (for instance when bile acid secretion is impaired due to simultaneous liver damage) is depressed, or (b) the *resorption of endotoxins* has been shown to increase (ruminal stasis, postpartum uterus, mastitis or laminitis caused by Gram-negative bacteria) (Smith, 1986; Peter et al., 1987; Lohuis, 1989; Andersen et al., 1990; Peter et al., 1990; Aiumlamai, 1991; Boosman et al., 1991; Cullor, 1992; Katholm and Andersen, 1992; Andersen et al., 1994*a*, 1994*b*; Sandholm and Pyörala, 1995). Generally, as the dosage of endotoxins increases, the latency time decreases, the peak effect becomes more

pronounced, and the duration of the effect protracted. Furthermore, individual susceptibility, age, pregnancy, charge of the endotoxin and the route of exposure/administration significantly influence the degree of distress (Smith, 1986; Lohuis, 1989; Giri et al., 1990; Jackson et al., 1990; Cullor, 1992; Jarlov et al., 1992; Sandholm and Pyörala, 1995).

Recently it has become known that endotoxins appear to have little direct toxic effects. The most severe clinical, haematological and blood chemical changes are indirect consequences, associated with a cascade of biological processes initiated by endotoxins (Westphal, 1975; Dinarello, 1983; Smith, 1986; Lohuis, 1989; Cullor, 1992; Sandholm and Pyörala, 1995). *In vitro* or *in vivo* exposure of neutrophils, phagocytic cells and platelets to endotoxin results in synthesis and release of increased amounts of many biologically active substances, i.e. various protein-like *cytokines* (α-tumor necrosis factor, TNF_α, interferons, as well as several members of the interleukin family), *biogenic amines* (histamine, serotonine), *oxygen radicals* (nitric oxide, hydrogen peroxide, superoxide) and — through the activation of the cyclooxygenase-2 (COX-2) and lipoxygenase enzyme systems — certain arachidonic acid (C20:4) derived lipid-like molecules such as *prostanoids* (prostaglandins including prostaglandin $F_{2\alpha}$, $PGF_{2\alpha}$ and prostaglandin I_2, PGI_2, as well as thromboxanes including thromboxane A_2, TxA_2; Table 1) and *leukotrienes*.

Table 1

The main effects of certain *prostanoids* liberated in endotoxin mediated diseases (after Ball et al., 1983; Emau et al., 1986; Giri et al., 1984; Fredriksson et al., 1985; Garcia-Villar et al., 1985; Giri et al., 1990)

Primary products	Their stable metabolites	Main effect(s)	
in the peripheral plasma			
PGI_2	6-keto-$PGF_{2\alpha}$	• vasodilatation ⇒ hypotension leading to shock	
$PGF_{2\alpha}$	PGFM	• in presence of susceptible CL: luteolysis	
		• stimulated contraction of smooth muscle in	uterus intestine airways ⇓
TxA_2	TxB_2	• vasodilatation ⇒ pulmonary hypertension ⇒	increased airway resistance
		• platelet aggregation	

The adequate release of these mediator substances is beneficial for the organism (moderate fever, generalised stimulation of the immune system, killing of microbes), their unbalanced overproduction, however, may lead to deleterious, harmful consequences (general depression, high fever, respiratory distress, disturbance in the motility pattern of the gastrointestinal — and urogenital? — tract, extreme polymorphonuclear leukopenia followed by leukocytosis, alterations in the carbohydrate, lipid and protein metabolism, redistribution of certain trace metals through the body, and in the most severe cases profuse diarrhoea, low blood pressure, disseminated blood clotting and at the end lethal shock). Almost all of these harmful consequences may be induced by experimental parenteral (intravenous or intramammary) administration of endotoxins or TNF_{α} alone (Smith, 1986; Lohuis, 1989; Andersen et al., 1990; Giri et al., 1990; Jackson et al., 1990; Cullor, 1992; Jarlov et al., 1992; Andersen et al., 1994*b*; Sandholm and Pyörala, 1995).

Endocrine interactions and their possible reproductive consequences in endotoxin-mediated diseases

Both the *in vivo* liberation and the experimental/accidental administration of endotoxin are followed by characteristic endocrine events and — at least under certain conditions — by metabolic and/or reproductive consequences.

The *temporary activation of the COX-2 enzyme system* and its consequences have been extensively studied. The temporary activation of the COX-2 by endotoxins may lead to increased production of prostanoids including $PGF_{2\alpha}$, and its stabile metabolite (15-keto-13,14-dihydro-$PGF_{2\alpha}$, PGFM) which can cause luteolysis when $PGF_{2\alpha}$-sensitive corpus luteum (CL) is present on the ovary. This mechanism is capable of shortening of the oestrous cycle with a premature return to oestrus *in non-pregnant ruminants* and *Equidae* (but not in pigs, due to the 13-day long $PGF_{2\alpha}$-insensibility of their developing CL after ovulation). Clinical importance can be attributed to this phenomenon first of all in postpartum dairy cows in which the activation of the C20:4 cascade by endotoxins absorbed from the uterus and the subsequent increase in $PGF_{2\alpha}$ and PGFM levels (a) seem to inhibit the first ovulation and CL formation, and (b) may play a role in shortening the life-span of the first CL after calving. In endometritic cows and mares the latter phenomenon may occur also later in the postpartum period (Peter and Bosu, 1987, 1988; Cort et al., 1990; Gilbert et al., 1990; Peter et al., 1990; Stabenfeldt and Edqvist, 1990; Kindahl et al., 1992, 1996). Of greater importance is, however, what happens to *pregnant animals*. It is well known that abortion occurs commonly following Gram-negative bacterial infections in many species (in mice, pigs, mares and also in ruminants). How-

ever, the primary mechanism by which Gram-negative bacteria are capable of inducing abortion is not clearly understood in all cases. If complete luteolysis occurs due to the endotoxin-induced COX-2 activation and subsequent $PGF_{2\alpha}$ release, abortion will take place within 24–48 h. This abortifacient property of luteolysis can be completed by the oxytocin-like uterotonic character of $PGF_{2\alpha}$ (Garcia-Villar et al., 1985). Abortion due to endotoxin-induced $PGF_{2\alpha}$ release has been reported to occur in *goat* during the entire pregnancy and in the *sow* at least until day 80 of gestation (Fredriksson et al., 1985; Cort and Kindahl, 1986, 1990), e.g. in species in which the progesterone production of the primary CL is responsible for the maintenance of pregnancy during the entire gestation (Stabenfeldt and Edqvist, 1990). In *cows* the endotoxin exposure is also followed by a marked, dose-dependent increase in the plasma $PGF_{2\alpha}$ level with a subsequent drop in progesterone concentration (Giri et al., 1990). In cattle, however, the pregnancy is maintained by progesterone secreted exclusively by the CL only in the early stage of gestation. Later, in the more advanced gestation the progesterone from extraluteal sources (mainly from the placenta, but perhaps also from the adrenal cortex of the dam) has physiological importance in the maintenance of pregnancy (Stabenfeldt and Edqvist, 1990). Based on this phenomenon, although endotoxins may lead to abortion also in cows in all stages of gestation, this effect is more considerable in the first trimester than in the second or third trimester of pregnancy (Giri et al., 1990). The *mare*, however, is sensitive to endotoxin-induced abortion only up to about day 50–60, e.g. by the time (a) of achievement in function of secondary CLs continuously forming in the ovary for about 2 months after day 35–40 of gestation, and (b) of the onset of placental gestagen production (Daels et al., 1987; Daels et al., 1991*b*; Kindahl et al., 1991). It should also be pointed out in various species that sometimes endotoxins can result in only a temporary fall in progesterone production without causing abortion (Cort and Kindahl, 1986, 1990; Daels et al., 1987; Giri et al., 1990; Daels et al., 1991*b*; Kindahl et al., 1991; Kindahl et al., 1996). Otherwise, in addition to the luteolytic and uterotonic effects of $PGF_{2\alpha}$ other C20:4 derivations such as TxA_2, a potent platelet aggregator and vasoconstrictor as well as PGI_2, a known vasodilatator causing hypotension (Table 1) may contribute to the pathophysiological process resulting in abortion through their local effects directed to the feto-placental unit (Giri et al., 1990). In other cases endotoxin exposition is known to cause bleeding and necrosis in placental tissues, and if this damage is excessive enough, secondary harm to the fetus will naturally result through a non-endocrine mechanism (Kindahl et al., 1996). The inhibition of COX-2 activity by flunixin meglumine (and also by other nonsteroidal anti-inflammatory drugs) has been reported to counteract the $PGF_{2\alpha}$-mediated aborti-

facient effect of endotoxins in domestic mammals (Anderson et al., 1986; Cort and Kindahl, 1990; Daels et al., 1991*a*; Jarlov et al., 1992).

Fewer data are available in the literature on the other endotoxin-influenced endocrine mechanisms. It is known that the endotoxins resorbed into the circulation and/or the endotoxin-induced cytokines, mostly TNF_{α}, will provoke (a) the hypothalamic/adenohypophyseal CRH/ACTH and TRH/TSH and prolactin release with a subsequent increase in thyroid hormone and adrenocortical glucocorticoid (in most of the domestic mammals: cortisol) production, (b) as well as a some days long decrease in insulin-like growth factor (IGF)-1 and IGF-binding protein-2 level, (c) but only a short reduction in the growth hormone (GH) concentrations in the blood (Jackson et al., 1990; Elenkov et al., 1992; Shuster and Harmon, 1992; Sordillo and Peel, 1992; Bosu et al., 1995; Elsasser et al., 1995; Sandholm, 1995).

In laboratory animals and cattle this increased *glucocorticoid* release can be stimulated by experimental endotoxin or TNF_{α} administration also directly and not only through the CRH/ACTH mediated pathway (Elenkov et al., 1992; Bosu et al., 1995), perhaps due to the fact that great pathophysiological and clinical importance can be attributed to these hormones in the down-regulation of the local TNF_{α} production on the site of the inflammatory process. Under physiological conditions this feedback-like mechanism is thought to be responsible for the prevention of the harmful (sometimes life-threatening) overproduction of certain cytokines, mainly of TNF_{α}. After a single intravenous or intramammary administration of LPS, or in a simple case of Gram-negative mastitis in cattle with normal adrenocortical responsiveness, this increase in the plasma cortisol level starts already a few minutes after the beginning of the endotoxin effect, it reaches peak concentration after 3–4 h and does not last longer (except for fatal cases) than 24 h. By this time, however, the endotoxin-stimulated cytokine increase including the plasma level of TNF_{α} has already dropped again to basal level (Lohuis, 1989; Hardie and Krouse-Elliot, 1990; Jackson et al., 1990; Shuster and Harmon, 1992; Sordillo and Peel, 1992; Elsasser et al., 1995; Sandholm, 1995). If, however, a higher dose (2.5 μg/kg) of endotoxin was administered in the form of a 6-h long intravenous infusion, elevated cortisol levels were detected for a longer (about 60-h) period (Giri et al., 1990). The high cortisol level caused by endotoxins resorbed from the postpartum uterus has been reported to suppress the preovulatory LH surge and so it may be one of the factors resulting in delayed resumption of cyclicity and/or formation of anovulatory cysts in dairy cows affected by uterine complications some weeks after calving (Bosu and Peter, 1987; Peter et al., 1989). On the other hand, temporary hypocorticism is considered to be a quite common phenomenon in dairy cows in the first postpartum week, but actually only very few or no data are available in the

literature on its effect on the course of endotoxin-mediated diseases (Kolk, 1991; Thuróczy et al., 1996).

The endotoxin-induced TRH release has been reported to lead to a subsequent increase in thyroid hormone (thyroxine, T_4) and prolactin (PRL) production in cows (Jackson et al., 1990; Sandholm, 1995). This change in T_4 level may be important in the thermoregulation and also in the regulation of the metabolic consequences of endotoxin-mediated diseases (Sandholm, 1995). In cows an obvious increase in PRL production was observed only when endotoxin was injected intravenously and not by the intramammary route (Jackson et al., 1990), and it is supposed to play a role in the immunomodulation but not in the milk production of the udder. In sows, however, experimental endotoxin administration was capable of decreasing both the PRL level and milk production in the first week after farrowing, but not later (Smith and Wagner, 1984, 1985). In laboratory animals (rat), the high-dose infusion of endotoxin was demonstrated to decrease both the peripheral level of thyroid hormones and the TSH-induced T_4 responsiveness through a membrane-damaging effect of LPS. Pretreatment with radiodetoxified endotoxin was proven to be an effective method in the prevention of this detrimental endocrine consequence (U. Nagy et al., 1983; Bertók and U. Nagy, 1984; U. Nagy and Bertók, 1990). A similar observation, however, has not yet been reported in farm animals.

The endotoxin-induced alterations in IGF-1, IGF-binding protein-2 and GH levels — and presumably also in glucocorticoid and T_4 concentrations — may be responsible for the LPS-related metabolic events shifting the metabolism from an anabolic to the catabolic state (Kenison et al., 1991; Clemmons and Underwood, 1992; Elsasser et al., 1994; Elsasser et al., 1995; Sandholm, 1995). Significant and chronic decreases in plasma concentrations of IGF-1, which are highly correlated with the nitrogen status of animals, have been associated with the progression of disease stress (Elsasser et al., 1988; Elsasser et al., 1989). In pregnant cows a 6-h long intravenous endotoxin infusion of 1 or 2.5 μg/kg caused an initial hyperglycaemia for 1–3 h which was followed by a gradually developing hypoglycaemia persisting as long as 18 and 30 h in the low and high dose groups, respectively. In the high dose group also elevated lactic acid levels were found for about 48 h. Endotoxin infusion at both doses caused a preferential mobilisation of oleic acid from adipose tissue, and also had some effects on the mobilisation of palmitic and stearic acids during the post-infusion periods (Giri et al., 1990). In lambs a catabolic effect in protein metabolism was attributed to endotoxin-induced TNF_α increase which might be prevented by IGF-1 pretreatment (Douglas et al., 1991). Although in the literature no data have been found to support this idea, the above mechanisms may have clinical relevance in the pathogenesis of the secondary ketosis in postpartum dairy cows.

Endotoxin-associated mastitis as a factor probably influencing reproductive performance in dairy cows during the early lactation

In well-managed dairy herds producing bulk milk with low (< 150 000–250 000) somatic cell count (SCC) in the first 3–4 postpartum weeks a significantly higher ratio of cows has been reported to suffer from acute mastitis than later in the lactation. The predominant part of these cases is caused by opportunistic bacteria including endotoxin-containing Gram-negative strains such as *E. coli*, sometimes *Klebsiella* spp., and others (Hogan et al., 1989; Schukken et al., 1989; Green et al., 1996; Miltenburg et al., 1996). The risk for early postpartum mastitis can be coupled with retained fetal membrane and subsequent putrid endometritis (Schukken et al., 1988). In spite of the known pathophysiological principles (Giri et al., 1984; Anderson et al., 1986; Katholm and Andersen, 1992; Sandholm, 1995; Sandholm and Pyörala, 1995) only very little is known about the clinical and reproductive importance of the above-mentioned endocrine consequences of endotoxin-associated (toxic) mastitis under practical (farm) conditions. Moore et al. (1991) reported that in a herd where *E. coli* was the predominant mastitis pathogen, the cows were almost twice as likely to have an altered inter-oestrus interval following an episode of clinical mastitis as their herdmates without clinical mastitis. Similar alteration in the inter-oestrus interval was not observed in a *Staphylococcus aureus* affected herd. However, further studies are required to confirm the supposed effect of Gram-negative mastitis on ovarian function, in which relevant endocrine methods should be used to follow up these consequences.

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THERAPEUTIC EFFICACY OF DOXYCYCLINE AGAINST EXPERIMENTAL *PASTEURELLA MULTOCIDA* INFECTION IN BROILER CHICKENS

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The efficacy of doxycycline was investigated in two sets of experiments. In the first experiment 40, in the second experiment 60, hence altogether 100 five-week-old Ross broilers of both sexes were used. The birds were randomly allocated into groups (A and B in experiment 1; A, B and C in experiment 2) of 20 birds in each. All birds were infected intramuscularly with approx. 2×10^3 colony forming units of *Pasteurella multocida* strain X-73 (serotype A:1). Birds in groups A were non-medicated controls. Chickens in groups B were given doxycycline via the drinking water at a dose of 10 mg/kg body weight for 5 days, while group C was treated with chlortetracycline at a dose of 20 mg/kg body weight for 5 days. The trial lasted for 9 days, then the surviving chickens were sacrificed. Clinical symptoms, number of deaths, post mortem lesions and bacteriological findings were recorded using a special score system. Acute fowl cholera developed in broilers within a few hours after infection, as evidenced by the clinical symptoms, the high mortality rate (90% of the birds died within 4 days after infection), the pathological lesions and the recovery of *P. multocida* from the challenged birds. Doxycycline reduced the number of deaths (30% and 5% of birds died in experiments 1 and 2, respectively) and the severity of the clinical symptoms, and *P. multocida* could be re-isolated only from one of the survivors. In contrast, chlortetracycline slightly influenced the mortality; however, it delayed death and reduced the severity of clinical symptoms. These data indicate that doxycycline is highly effective for the treatment of experimental pasteurellosis in chickens.

Key words: *Pasteurella multocida*, fowl cholera, doxycycline, chicken

Fowl cholera (FC), caused by the bacterium *Pasteurella multocida*, is a common disease of domesticated and wild birds that gives rise to severe economic losses in the poultry industry world-wide. This infectious disease usually appears as an acute septicaemia with high morbidity and mortality especially among chickens and turkeys but goose and duck flocks are also frequently affected. In addition to the septicaemic form, chronic conditions may occur, re-

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sulting in the development of a carrier state (Rhoades and Rimler, 1989; Campi et al., 1990; Rhoades and Rimler, 1991; Hafez et al., 1992).

Despite many efforts to produce effective attenuated or killed vaccines, the commercial vaccine preparations available for immunisation have proven only moderately effective in preventing losses (Rhoades and Rimler, 1991).

Sulphonamides and some antibiotics are being used to treat or prevent the disease. Very often the results of treatment with antimicrobials are of limited value because of the resistance of *P. multocida* strains to the available drugs or due to the poor pharmacokinetics of the drug formulation used. Therefore, search for new effective drugs is needed.

Doxycycline has been successfully used to combat various bacterial infections in humans. Data on the clinical use of doxycycline in mammals are rather limited, and only two publications have been found on the efficacy of this drug against experimental colibacillosis in chickens (George et al., 1977; Goren et al., 1988).

The purpose of the present study was to evaluate the effectiveness of doxycycline (DOC) in experimental *P. multocida* infection in broilers in comparison with chlortetracycline (CTC).

Materials and methods

Experimental birds and housing

A total of 100 Ross broiler chickens of both sexes, aged 5 weeks, were used for evaluating the efficacy of doxycycline and chlortetracycline on FC under controlled experimental conditions. They were obtained from a non-vaccinated flock with no clinical history of the disease. The birds weighed 1150–1720 g at the beginning of the experiment.

The experimental chickens were placed into batteries. Room temperature (21–22 °C) and humidity were adjusted to meet the requirements of the birds. Water and feed were offered from drinking- and feeding-troughs. A full-day light regimen was applied throughout the experiment.

The chickens were kept on a commercial broiler diet that did not contain any drug, anticoccidial or growth promoter. Feed was provided *ad libitum* throughout the experiment.

Experimental design

Experiment 1. A total of 40 broilers were randomly divided into two groups of 20 birds each, weighed and labelled individually. Chickens in group A were infected non-medicated controls. Broilers in group B were infected and treated with DOC. Treatment was started immediately after infection.

Experiment 2. A total of 60 broilers were randomly divided into three groups of 20 birds each, weighed and labelled individually as in experiment 1. Broilers in group A were infected non-medicated controls. Chickens in groups B and C were infected and treated with DOC and CTC, respectively. Treatment was started immediately after infection.

Infection procedure

Pasteurella multocida strain X-73 (serotype A:1) was used for infection (ref. Dr. R. B. Rimler, NADC, Ames, Iowa, USA). Encapsulated colonies of the strain were cultivated on dextrose starch agar (DSA) for 18 h at 37 °C and suspended in phosphate buffered saline (PBS), pH 7.2. The turbidity of the suspension was adjusted to obtain a density visually comparable to McFarland No. 1 turbidity standard which contained approximately 2.5×10^8 colony forming units (cfu)/ml. From this, an appropriate dilution was made and a dose of approximately 2,000 cfu was inoculated intramuscularly into the breast muscle in a volume of 1 ml.

Treatment procedure

Experiment 1. Doxycycline hydrochloride (as hyclate, Doxyvit 100 SP, Lavet Ltd., Budapest, Hungary) was given via the drinking water at a dose of 10 mg DOC per kg body weight for 5 consecutive days. The amount of DOC was calculated on the basis of the initial body weight of the birds on day 0 and then it was adjusted considering an average daily weight gain of 50 g/bird for the surviving chickens. Consequently, the dose of 10 mg/kg body weight was ensured during the whole treatment period. Medicated water was freshly prepared every morning and offered to birds in a defined volume which is generally consumed by healthy chickens in 4–6 h (pulse dosage regimen).

Experiment 2. DOC was applied as described in experiment 1. Chlortetracycline hydrochloride (Aureomycin 5.5 SP, Cyanamid, UK) was also given via the drinking water at a dose of 20 mg CTC per kg body weight for 5 consecutive days. The calculation of the drug dose and the way of application of the medicated water was the same as described for DOC.

Evaluation of the treatment

For evaluating the efficacy of the treatment, clinical symptoms, pathological lesions and bacteriological infestation of the birds were considered.

Clinical symptoms were recorded daily (at 8:00 a.m.) for 9 days and evaluated according to the following score system:

Symptom	Score number
Nasal discharge	1
Lameness	2
Somnolence	2
Weakness	3

To compare the clinical signs observed during the treatment period (days 1–5) and the follow-up period (days 6–9) as well as between groups the following calculation was made. The daily scores of individual chickens in the group were summed up and divided by the actual number of birds. The daily mean scores obtained in this way were added together separately for the treatment period and the follow-up period, and the sum was divided by the number of days in the period.

Necropsy. All birds that died or were euthanised were examined for gross pathological lesions. The severity of lesions was scored as follows:

Lesion	Score number
Mucus in the nasal cavity	1
Swollen liver	2
Subepicardial haemorrhage	2
Foci of necrosis in the liver	2
Foci of necrosis in the spleen	1
Fibrinous exudate on the visceral membranes	1

For comparison, the sum of scores was divided by the number of birds in the group.

Bacteriological culturing

In experiment 1 re-isolations of the challenge strain were carried out from the liver and heart of birds that died or were sacrificed, while in experiment 2 the isolation procedure was extended to the bone marrow of chickens that survived and were sacrificed at the end of the experiment. Using a standard loop, approximately equal portions of the specimens were streaked on blood agar plates and the results were read after incubation at 37 °C for 18 h. The density of growth was described with crosses: confluent pure culture (3), countable colonies (2), less than 50 colonies (1), no growth (0). These marks were summed up to express the bacterial infestation.

Determination of the minimum inhibitory concentration (MIC)

For strain X-73 of *P. multocida* the MIC of DOC (Nedermed, The Netherlands) and CTC (Cyanamid, UK) was determined by the twofold serial agar dilution method performed according to NCCLS standard procedures (NCCLS, 1993).

Results

Experiment 1. The number of deaths, the severity of lesions in terms of average scores, the clinical symptoms observed during the first (days 1–5) and second (days 6–9) period of experiment, and the results of bacteriological examinations in the control and the DOC-treated groups are summarised in Table 1.

Table 1

Therapeutic effect of doxycycline on experimental fowl cholera in chickens

Group	Mortality	Severity of lesions at necropsy	Clinical symptoms		Bacterial infestation
			Days 1–5	Days 6–9	
A (control)	19/20[a]	5.25[b]	2.20[c]	0[c*]	114[d]
B (treated)	6/20	2.20	0.42	0.39	35

[a]Number of chickens that died/number challenged; [b]Total scores/number of birds; [c]Sum of the daily mean scores divided by the number of days indicated; [d]The figure includes the total scores obtained from each bird during the whole observation period. The scores reflect the density of bacterial growth: confluent growth 3, more than 50 colonies 2, less than 50 colonies 1; isolation was performed from liver and heart and the marks were summarised; [*]One chicken survived only that showed no clinical sign

Acute FC developed in broilers of the control group (Group A) within a few hours after infection, as evidenced by the clinical symptoms, the high mortality rate (19 out of 20 chickens died within two days after infection), the pathological lesions, and the recovery of *P. multocida* from the challenged birds. In contrast, only 6 chickens died of pasteurellosis in the group treated with DOC, 1, 4 and again 1 death occurred on days 2, 3 and 7 after challenge, respectively. The lesions were not as pronounced as in group A. The very low scores of the clinical signs (0.42 and 0.39 for the first and second observation period, respectively) indicated that only some of the birds were affected and the symptoms were mild. Re-isolation attempts from the liver and heart of sacrificed birds gave negative result but cultivation from broilers that died yielded a pure and dense growth.

Experiment 2. Seventeen out of the 20 infected non-treated broiler chickens died. The majority (16 birds) of losses occurred within 4 days after infection. The clinical symptoms and pathological lesions were characteristic of FC. In Group B (DOC) only one bird died during the observation period and slight signs of FC could be seen in another chicken. These data (Table 2) clearly showed that oral medication with DOC was highly effective in reducing mortality and clinical symptoms in infected birds. At the end of the experiment *P. multocida* could be re-isolated in very low numbers (the yield was less than 50 colonies) from the liver and heart of a single chicken, while in the rest of the birds the bacteriological examination was negative even from bone marrow samples. In contrast, in the CTC-treated group (Group C) the mortality was only slightly reduced; however, the treatment prolonged the survival of the chickens and resulted in somewhat less pronounced clinical symptoms (Table 2, note data on clinical symptoms between days 6 and 9). Negative bacteriological findings including the bone marrow samples were obtained only in three broiler chickens.

Table 2

Therapeutic effect of treatment on experimentally induced fowl cholera in chickens

Group	Mortality	Severity of lesions at necropsy	Clinical symptoms		Bacterial infestation
			Days 1–5	Days 6–9	
A (control)	17/20[a]	5.90[b]	1.97[c]	3.04	111[d]
B (DC treated)	1/20	0.35	0.062	0.0	7
C (CTC treated)	15/20	5.15	1.81	1.69	86

DC = doxycycline; CTC = chlortetracycline; [a]Number of chickens that died/number challenged; [b]Total scores/number of birds; [c]Sum of the daily mean scores divided by the number of days indicated; [d]The figure includes the total scores obtained from each bird during the whole observation period. The scores reflect the density of bacterial growth: confluent growth 3, more than 50 colonies 2, less than 50 colonies 1; isolation was performed from liver and heart and the marks were summarised

MIC of antibacterial agents

The MICs of DOC and CTC for the challenge strain of *P. multocida* X-73 were 0.06 and 0.5 μg/ml, respectively.

Discussion

Sulphonamides and some antibiotics have been used to treat or control fowl cholera with varying results (Rhoades and Rimler, 1991). The success of medication depends, among others, on factors such as sensitivity of the pathogen to the antimicrobials used, pharmacokinetic behaviour of the drug, i.e. the rate and extent of absorption, the drug concentrations attained at the site where pasteurellas are localised, the residence time of the drug in the tissues, etc. In acute cases the outcome of the treatment is highly influenced by the stage of the disease at which treatment is initiated. In addition, the route of drug administration and the pharmaceutical properties of the preparations also play a role in the effectiveness of treatment. As affected birds do not eat but generally they still drink, it is advisable to administer the drug via the drinking water. The intramuscular application of penicillin, streptomycin alone or in combination and some other antibiotics proved to be effective against experimental FC if adequate drug intake was ensured (Bierer, 1962). However, this mode of application is laborious and may cause transmission of the pathogen; thus, it is not practical in poultry flocks.

Variability in the antimicrobial susceptibility of *P. multocida* strains should be considered during therapy (Rhoades and Rimler, 1991). Resistance of this pathogen to penicillin, tetracyclines and sulphonamides is thought to be uncommon, but the present state of sensitivity has not been fully surveyed. Recent data indicate that the incidence of strains of *P. multocida* resistant to traditional drugs is probably higher than expected (Hirsch et al., 1985; Reece and Cole, 1985). The value of DOC in the treatment of FC has not been investigated.

Acute FC developed within a few hours after infection in the control groups, causing 95% and 85% losses in experiments 1 and 2, respectively. Among the control birds death occurred within 24 h of challenge. The clinical symptoms and pathomorphological lesions were similar to, or even more pronounced than those seen in the natural outbreaks. *P. multocida* could be readily isolated from liver and heart.

According to the results obtained in this study, water medication with DOC proved to be highly effective against experimentally induced FC; however, some differences were found between the two sets of experiments. Compared with the controls, death losses were reduced from 95% to 30% in experiment 1 and from 85% to 5% in experiment 2. The disease was manifested in mild symptoms in the treated chickens of experiment 1, and 55% of the birds were affected. In experiment 2, DOC-treated birds showed practically no clinical signs. The differences in data (see Tables 1 and 2) between experiment 1 and experiment 2 can be explained either by the higher sensitivity of chickens used in experiment 1 to *P. multocida* or by the inevitable variation in the dose of chal-

lenge. The latter might have resulted from an inherent error of the dilution method. Since the cfu of the bacterial suspension used for challenge was rather low (approximately 2,000), even a slight bias could modify the outcome of the challenge. Consequently, both facts may have been involved in that the infection caused a somewhat higher rate of death among control and treated birds and the disease appeared in a more severe form in experiment 1 than in experiment 2.

The bacterial infestation differed remarkably among groups. In the DOC-treated group *P. multocida* could be isolated from the dead birds (experiments 1 and 2) and from a single survivor only (experiment 2). This finding is very important in respect of the carrier state of the pathogen.

In contrast, CTC at twice the dose of DOC slightly reduced the mortality and the severity of clinical symptoms but prolonged the time to death. Negative bacteriological results were obtained only in two chickens.

The unsatisfactory efficacy of CTC in the present experiment is inconsistent with some observations. For instance, Japanese quail infected with *P. multocida* were successfully treated with CTC; however, the treatment lasted for 10 days and the case was a natural outbreak (Glisson et al., 1989). CTC was also effective for preventing fowl cholera in turkeys exposed to *P. multocida* via the drinking water (Olson, 1977) which, of course, is not as serious a challenge as the intramuscular route. In conformity with our results, DOC was found to be more effective than CTC against *E. coli* infection in young chickens (George et al., 1977).

The superior efficacy of DOC over CTC in this study can be explained by the following considerations. In comparison with oxytetracycline and CTC, DOC possesses an expanded antimicrobial spectrum and improved activity (Aronson, 1980; Shaw and Rubin, 1986; Pijpers et al., 1989). The stronger *in vitro* activity of DOC against *P. multocida* was also corroborated in this study. Furthermore, due to its lipophilicity DOC favourably differs in pharmacokinetic properties from the older tetracyclines (Prescott and Baggot, 1993). In chickens, DOC administered directly into the crop was absorbed more rapidly and extensively, and attained several times higher plasma concentrations for a longer time than CTC (Semjén et al., 1994). The favourable pharmacokinetic characteristics of DOC in chickens were also confirmed by Anadon et al. (1993). Finally, drinking water medicated with DOC was fully consumed within 4–6 h while the intake of CTC lasted for 8–12 h or even longer. The intake of DOC within a short period of time might have resulted in much higher drug concentrations in the plasma and tissues than those attained after a protracted intake. Probably the difference in palatability could be the reason why the broilers were reluctant to consume the drinking water containing CTC.

This study indicates that DOC is a good candidate for the treatment of poultry against systemic bacterial infections such as pasteurellosis.

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INFLUENCE OF *ESCHERICHIA COLI* ENDOTOXIN INDUCED FEVER ON THE PHARMACOKINETICS AND DOSAGE REGIMEN OF OXYTETRACYCLINE IN CROSS-BRED CALVES

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The pharmacokinetics and dosage regimen of oxytetracycline were determined in healthy and febrile cross-bred calves following its single intravenous administration (10 mg kg^{-1}). Fever was induced by a single intravenous injection of *E. coli* endotoxin (1 μg kg^{-1} i.v.). The elimination half-life ($t_{½}$ β) and the apparent volume of distribution [$vd_{(area)}$] were slightly increased in febrile calves, as compared to healthy animals. The values of $t_{½}$ β and $Vd_{(area)}$ were 3.22 ± 0.20 h and 0.49 ± 0.02 L. kg^{-1} in healthy calves and 4.06 ± 0.32 h and 0.70 ± 0.09 L kg^{-1}, respectively, in febrile calves. An intravenous dosage regimen suitable for maintaining the minimum therapeutic plasma concentration of ≥ 2 μg/ml in febrile animals would be 10 mg kg^{-1} repeated at 12-h intervals.

Key words: Pharmacokinetics, oxytetracycline, fever, calves

Oxytetracycline, a routinely used broad-spectrum antibiotic is also effective in various infections caused by Rickettsia, Chlamydia and Mycoplasma (Dawson et al., 1971; Blood et al., 1983). There are several reports that the pharmacokinetics of antimicrobial agents are altered in disease conditions (Ladefoged, 1980; Guerrini et al., 1984; Quintiliani et al., 1984; Groothuis et al., 1980; Roy et al., 1992), hence the dosage regimen obtained in healthy subjects cannot be extrapolated to diseased animals to treat various infections. There is only limited information on the disposition kinetics of oxytetracycline in disease conditions (Ames et al., 1983; Burrows et al., 1986). Keeping this in view, in the present study the influence of *E. coli* endotoxin induced fever on the pharmacokinetics and dosage regimen of oxytetracycline was determined in cross-bred calves.

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Materials and methods

Animals and treatment

The experiments were performed on ten clinically healthy cross-bred calves weighing 80–100 kg. The animals were maintained for 15 days before the commencement of experiment and were provided green fodder and water *ad libitum.* The average day temperature in the animal shed was approximately 38 °C. They were divided into two groups of five animals each. In animals of group I, oxytetracycline (Terramycin, Pfizer India Ltd.) was administered as a single intravenous injection into the jugular vein at the dose rate of 10 mg/kg body weight. In animals of group II fever was induced by a single intravenous injection of *E. coli* endotoxin (Sigma Chemical Company, USA) at the dose rate of 1 μg/kg body weight. Once fever developed, oxytetracycline was injected at the same dose rate as in animals of group I.

Collection and analysis of samples

In both groups of animals blood samples were collected from the contralateral jugular vein at 1, 2.5, 5, 7.5, 10, 15, 30, 60, 90, 120, 240, 360, 480, 600, 720, 840 and 960 min after drug administration. Plasma was separated by centrifugation at 3000 *g* for 15 min at room temperature and stored at –20 °C until analysed. The concentration of oxytetracycline was measured in the plasma by a microbiological assay using *Bacillus cereus* var. *mycoides* (ATCC 11778) as test organism (Arret et al., 1971).

To determine the disposition kinetics and dosage regimen of oxytetracycline, the data on plasma concentration–time profile of each experimental animal was analysed separately (Baggot, 1977; Gibaldi and Perrier, 1982). The values of fc and T/P ratio were also calculated by the following equations:

$$fc = \beta/k_{el}$$
$$T/P = (1/fc) - 1$$

The significant difference between the two groups of animals was calculated by using paired *t*-test.

Results and discussion

In animals of group II, a single i.v. injection of *E. coli* endotoxin (1 μg/kg) induced fever within 45–60 min. Fever persisted for up to 10–12 h. During the experimental period, the body temperature of febrile animals ranged between 39.2 ± 0.33 to 40.4 ± 0.28 °C (102.6 ± 0.56 to 104.8 ± 0.51 °F). The plasma concentrations of oxytetracycline in both groups of calves following a single intravenous

injection are presented in Table 1. At 1 min the peak plasma concentration was 104.8 ± 3.23 and 115.8 ± 6.27 μg/ml in healthy and febrile calves, respectively. In healthy calves oxytetracycline was detected only up to 12 h (1.76 μg/ml), however, in febrile calves the drug was detected in traces (1.09 μg/ml) up to 16 h of injection. Clarke et al. (1974) reported that following i.v. injections of oxytetracyline to normal and pneumonic cattle, the blood levels attained were significantly higher in pneumonic cattle than in normal animals.

Table 1

Plasma levels (μg ml^{-1}) of oxytetracycline after its single intravenous administration (10 mg/kg) in healthy and febrile calves

Time after drug administration (min)	Healthy	Febrile[a]
1	104.8 ± 3.23	115.8 ±8.27
2.5	93.2 ± 2.41	98.2 ± 4.98
5	50.3 ± 0.98	50.2 ± 1.90
7.5	47.1 ± 2.29	46.1 ± 1.79
10	45.5 ± 1.11	44.4 ± 1.58
15	41.6 ± 1.65	40.6 ± 0.66
30	18.1 ± 0.64	19.1 ± 0.42
60	16.0 ± 0.26	16.5 ± 0.67
90	15.6 ± 0.30	15.7 ± 0.44
120	8.41 ± 0.31	8.02 ± 0.15
240	7.28 ± 0.36	8.02 ± 0.15
360	3.84 ± 0.15	3.19 ± 0.22
480	3.30 ± 0.15	2.93 ± 0.16
600	2.75 ± 0.10	2.74 ± 0.17
720	1.76	1.26 ± 0.08
840	ND	1.18 ± 0.08
960	ND	1.09

Values given are mean ± SE of five animals; [a]Fever was induced by a single i.v. injection of *E. coli* endotoxin (1 μg/kg); ND = not detected

The concentration time curve of oxytetracycline was best described by an open 2-compartment model.

Various pharmacokinetic parameters of oxytetracycline were calculated and presented in Table 2. In healthy calves, the elimination half-life and volume of distribution of oxytetracycline were 3.22 ± 0.20 h and 0.49 ± 0.02 L Kg^{-1}, respectively. In other investigations the values of $t_{½}$ β and $Vd_{(area)}$ in dairy cows have been reported to be 4.1 ± 0.6 h (Ziv and Sulman, 1974) and 2.9 ± 0.7 $L.kg^{-1}$

(Prasad et al., 1987), respectively. The high values of α (12.6 ± 3.22 h^{-1}) and T/P ratio (5.18 ± 0.71) revealed that oxytetracycline is rapidly distributed in various body fluids and tissues. In contrast, as compared to the present results, higher values of $t_{½}$ β (11.84 ± 1.7 h), $Vd_{(area)}$ (3.34 ± 0.33 L kg^{-1}) and Vc (0.97 ± 0.25 L kg^{-1}), and lower values of α (0.41 ± 0.25 h^{-1}), K_{12} (0.21 ± 0.09 h^{-1}), K_{21} (0.14 ± 0.09 h^{-1}) and k_{el} (0.14 ± 0.02 h^{-1}) of oxytetracyline have been reported in calves (Ames et al., 1983). The reason for the marked difference between the kinetic parameters of oxytetracycline in the present paper and those reported by Ames et al. (1983) is not clear; however, climatic conditions and other factors may be implicated. Climatic temperature has been established to influence the pharmacokinetics and dosage regimen of antibacterial drugs in a particular species (Heinze and Weirowski, 1991; Ginneken et al., 1991). Caligiuri et al. (1990) have reported that in tortoises, kept in warmer climate (30 °C), the metabolism, clearance, MRT and AUC of amikacin (an aminoglycoside antibiotic) was twice as fast as in tortoises kept at lower temperatures (20 °C).

Table 2

Pharmacokinetics of oxytetracycline after its single intravenous administration (10 mg/kg) in healthy and febrile calves

Parameter	Unit	Healthy	Febrile[a]
Cp^{o}	μg ml^{-1}	138.9 ± 15.03	130.8 ± 16.4
A	μg ml^{-1}	120.9 ± 15.5	118.1 ± 17.1
α	h^{-1}	12.6 ± 3.22	10.0 ± 2.27
$t_{½}α$	h	0.07 ± 0.01	0.11 ± 0.04
B	μg ml^{-1}	18.0 ± 0.57	12.9 ± 1.52[b]
β	h^{-1}	0.22 ± 0.02	0.18 ± 0.01
$t_{½}β$	h	3.22 ± 0.20	4.06 ± 0.32
k_{12}	h^{-1}	9.61 ± 2.91	7.65 ± 1.94
k_{21}	h^{-1}	1.79 ± 0.30	0.99 ± 0.14
k_{el}	h^{-1}	1.49 ± 0.17	1.55 ± 0.24
$t_{½}$ k_{el}	h	0.50 ± 0.06	0.50 ± 0.09
AUC	μg ml^{-1} × h	94.54 ± 4.89	87.78 ± 7.51
$Vd_{(area)}$	L kg^{-1}	0.49 ± 0.02	0.70 ± 0.09
Cl_B	ml kg^{-1} × h^{-1}	107.2 ± 5.94	117.2 ± 8.35
fc	ratio	0.17 ± 0.01	0.13 ± 0.03
T/P	ratio	5.18 ± 0.71	8.44 ± 1.87

Values given are mean ± SE of five animals; [a]Fever was induced by a single i.v. injection of *E. coli* endotoxin (1 μg/kg); [b]Significantly ($P < 0.05$) different as compared to its corresponding value in healthy animals

A comparison between pharmacokinetic parameters obtained in febrile and healthy animals revealed that the elimination half-life (4.06 ± 0.32 h) was slightly increased during fever as compared to 3.22 ± 0.2 h in healthy animals. Similarly, $Vd_{(area)}$ was also slightly increased in febrile calves (0.70 ± 0.09 L kg^{-1}) as compared to healthy animals (0.49 ± 0.02 L kg^{-1}). Ames et al. (1983) have reported a significant increase in the volume of distribution and elimination half-life of oxytetracycline in pneumonic cattle as compared to healthy animals. Similarly, prolonged $t_{½}\beta$ of oxytetracyline in goats with *Ehrlichia phagocytophila* induced fever as compared to healthy animals has also been established (Anika et al., 1986). There was no significant effect of *E. coli* endotoxin induced fever on other kinetic parameters, viz. distribution rate constant (α), distribution half-life ($t_{½}\ \alpha$), k_{el}, AUC, K_{21} and volume of central compartment (Vc).

The major objective of the present investigation was to calculate and modify the dosage regimen of oxytetracycline for the treatment of fever. The dosage regimen of drug predicted on the basis of pharmacokinetic study is intended only as a guideline and the effectiveness of a drug in clinical conditions still remains to be verified. For oxytetracycline, the value of minimum effective concentration ranged between 1.6–4 μg ml^{-1} (Standiford, 1979) but a concentration of ≥ 2 μg/ml is considered to be the adequate minimum effective plasma concentration, which is effective against the majority of bacteria (Baggot, 1977).

After choosing a suitable and convenient dosage interval, the priming (D) and maintenance (D^1) doses are calculated by following equations, respectively.

$$D = MEC \times Vd_{(area)} (e^{\beta\tau})$$
$$D^1 = MEC \times Vd_{(area)} (e^{\beta\tau}-1)$$

By putting the values of pharmacokinetic constants obtained in febrile calves, at the minimum effective plasma concentration of ≥ 2 μg/ml, the priming and maintenance doses would be 11.4 and 10 mg/kg , respectively, at a 12-h interval or under field conditions oxytetracyline can be administered at a dose rate of 10 mg/kg at 12-h intervals.

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THE INFLUENCE OF REPRODUCTIVE STAGE ON THE SELENIUM STATUS OF SHEEP IN A LOW-SELENIUM REGION

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The selenium status of sheep was evaluated during the reproductive stage in a region of low selenium level. Serum selenium concentration, whole blood glutathione peroxidase activity (GSH-Px), which is a good indicator of protection against oxidative damage, as well as the activities of creatine kinase (CK) and aspartate aminotransferase (AST), the plasma indicators of muscle damage, were evaluated in a group of ewes during gestation and lactation and in their lambs. The selenium requirements of ewes were found to increase during lactation. There were no differences in GSH-Px activity between the experimental and the control groups throughout the reproductive stage. In the second half of pregnancy GSH-Px activity was subnormal. In spite of this, no evidence of existing pathologic conditions associated with selenium deficiency was found, since the muscle markers CK and AST were within the normal range. In the same way, no distinct symptoms of nutritional myopathy were observed in the lambs, suggesting that the low selenium level found in the ewes did not cause alterations in their development.

Key words: Serum selenium, glutathione peroxidase, muscle enzymes, reproductive stage, sheep

The biochemical role of selenium in animals was elucidated by Rotruck et al. (1973) as a component of the enzyme glutathione peroxidase (GSH-Px, E.C. 1.11.1.9). GSH-Px functions in cellular oxidation-reduction reactions to protect the cell membranes from oxidative damage caused by free radicals and peroxides (Flohe et al., 1973).

White muscle disease was documented as a selenium and vitamin E deficient condition in sheep three decades ago (Muth et al., 1958; Hogue et al., 1962). For some years increasing attention has been paid to the influence of selenium on reproductive performance. There is substantial evidence that pregnant ewes require more selenium than non-pregnant ones. For this reason, selenium supplementation of the maternal diet during pregnancy and lactation is crucial for the prevention of selenium deficiency in several species of farm animals

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(Zachara et al., 1992; Donald et al., 1993; Sáez et al., 1996). This is because large amounts of free radicals occur as intermediate products in the metabolic pathways of developing organisms. In a selenium-deficient ewe, the antioxidative systems can be overloaded. If free radicals are not eliminated, these metabolic pathways will easily be deregulated, bringing about pathological conditions, the final consequence of which will be the inability to maintain reproduction (Bostedt and Schramel, 1990).

The objective of this experiment was to monitor the selenium status of sheep in a low-marginal selenium area (López Alonso, 1995) during the reproductive stage, and to determine whether low selenium status could affect the development of lambs. For this purpose, serum selenium concentration, blood GSH-Px activity (a good indicator of protection against oxidative damage), as well as the plasma indicators of muscle damage, creatine kinase (CK) and aspartate aminotransferase (AST) activities were evaluated.

Materials and methods

Animals

Twenty native ewes of the Gallega breed, ranging from 2 to 4 years of age, were selected from a flock in a low-selenium area of Lugo (NW Spain). The ewes were kept on natural pastures (a mixture of white clover and rye-grass) during the day, and offered local hay (from the same pastures) *ad libitum* at night. The calculated Se content of the diet varied between 0.02 and 0.04 mg/kg of selenium (on dry matter basis).

The animals were randomly allotted to 2 equal groups. The experimental group comprised the ewes that were going to be pregnant and lactating during the experiment, and the control group comprised 10 non-pregnant animals. The experimental ewes were subjected to a synchronisation process to harmonise their physiological condition (Britt, 1989). Pregnancy was diagnosed at the second month by ultrasonography.

Blood samples

Blood samples were drawn by jugular venepuncture at monthly intervals during pregnancy, 15 days before parturition, immediately after parturition, and weekly during lactation. At the same times, samples were collected also from the control group and from the lambs.

Each sample was placed into heparinised tubes and tubes for serum separation, and kept refrigerated for transportation to the laboratory. Whole heparinised blood was assayed for GSH-Px activity, and serum was analysed for

selenium, CK and AST activities. GSH-Px, CK and AST activities were determined in fresh material (within 12 h of sampling), while serum selenium analyses were performed at the end of the experiment using samples that had been frozen (–20 °C).

Analytical determinations

Selenium concentration in blood sera was determined by hydride generation atomic absorption (HD-AAS) (Norheim and Haugen, 1986).

GSH-Px was measured with a commercial kit (Ransel®, Randox, UK) on the basis of the method of Paglia and Valentine (1967). Enzyme activity was expressed in international units per gram of haemoglobin (IU/g Hb). Haemoglobin was determined by the standard cyanmethaemoglobin method (Hainline, 1958).

Serum creatine kinase (CK) and aspartate aminotransferase (AST) activities were expressed in international units per litre (IU/l) and were assayed using kinetic procedures with commercial reagents (Knickerborcker, SAE; Cromatest Reagents).

Statistical analyses

One-way analysis of variance (Fisher's F test) was used to test for changes in the parameters analysed, and for differences between the two groups (Snedecor and Cochran, 1978). Differences were considered significant at $p < 0.05$.

Results

Serum selenium concentrations in the ewes and in their lambs are shown in Fig. 1. In ewes this parameter showed a similar evolution during gestation, the decrease of serum Se concentration in the experimental group at the beginning of lactation ($p < 0.05$) being the only significant difference detected between the groups. This decrease of Se in maternal blood was accompanied by an increase of serum Se in the lambs during the first week of life.

There were no significant differences between experimental and control ewes in blood GSH-Px activity during the whole reproductive stage (Table 1). GSH-Px activity decreased drastically, by 50% in both groups at a time which corresponded with the third month of pregnancy for the experimental group ($p < 0.001$). Subsequently there was a slight recuperation, parallel in both groups. However, this low GSH-Px activity, evident especially during late gestation, was not reflected in the activity of the muscle enzymes CK and AST (Table 1). Although in some cases their activity was high, the mean values were usually within the physiological range.

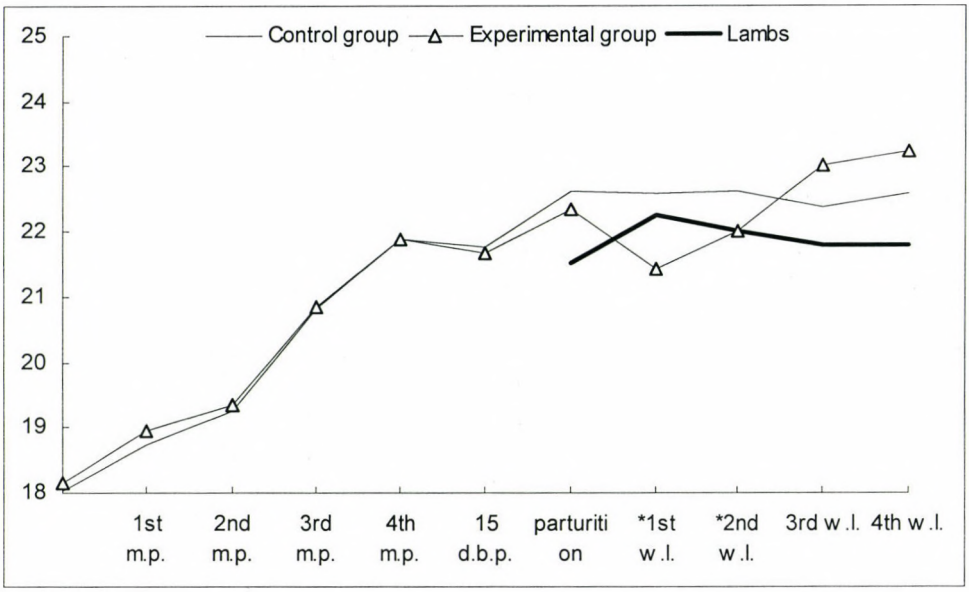

Fig. 1. Changes of serum Se (ng/g) in ewes and lambs during the reproductive stage (*denotes a significant difference of $p < 0.05$ between experimental and control groups; m. p.: month of pregnancy; d. b. p.: days before parturition; w. l.: week of lactation)

GSH-Px activity as well as CK and AST activities of the 15 lambs born in the experimental group are presented in Table 2. When blood samples were collected for clinical analysis, no evidence of existing muscle disease was found in the lambs. This finding is consistent with blood GSH-Px as well as serum CK and AST activities which are within the normal range during the first month of life.

Discussion

Evidence that ewes were maintained in a low-marginal Se area during this study was supported by the low serum selenium concentration (Dixon and Dunner, 1983; McDowell et al., 1987), and the marginally deficient GSH-Px activity (Mañé et al., 1991; Sáez et al., 1995).

Although most authors agree that females require more selenium during the reproductive stage, there are contradictory views on the behaviour of Se concentration and GSH-Px activity during that time. In this sense, Segerson et al. (1986) have shown that in unsupplemented ewes the serum Se concentration increased between the mating season and lambing, while Zachara et al. (1989), in a similar situation, have indicated a continuous decrease of whole blood Se and a constant but critically low plasma Se level from the mating season up to

Table 1

Changes in the activities of whole blood glutathione peroxidase (IU/g Hb) and serum creatine kinase and aspartate aminotranferase (IU/l) in the experimental and control groups during the reproductive stage (expressed as mean ± SEM)

	Gestation						Parturition	Lactation			
	Basal	1st m. g.	2nd m. g.	3rd m. g.	4th m. g.	15 d. b. p.		1st w. l.	2nd w. l	3rd w. l.	4th w. l.
						Experimental group					
GSH-Px	77.15 ± 13.62	93.30 ± 24.73	80.31 ± 8.10	47.95 ± 5.84	44.60 ± 6.98	40.25 ± 11.32	57.31 ± 12.89	58.28 ± 9.14	75.44 ± 9.11	74.63 ± 13.96	75.44 ± 9.02
CK	76.86 ± 9.42	61.51 ± 7.02	59.45 ± 7.63	122.26 ± 15.8	74.02 ± 10.80	88.01 ± 18.15	76.85 ± 10.86	85.46 ± 15.39	68.15 ± 6.63	52.10 ± 5.40	57.21 ± 3.37
AST	82.87 ± 4.62	83.87 ± 5.51	74.52 ± 4.02	73.50 ± 5.27	68.37 ± 3.58	64.75 ± 2.78	67.50 ± 5.88	63.87 ± 4.15	31.37 ± 4.40	50.75 ± 1.92	52.50 ± 1.96
						Control group					
GSH-Px	71.16 ± 5.84	76.92 ± 17.79	100.70 ± 15.0	38.71 ± 7.86	55.19 ± 10.13	59.77 ± 14.34	77.09 ± 12.16	70.61 ± 14.41	69.47 ± 12.98	103.60 ± 28.4	83.05 ± 17.67
CK	95.64 ± 21.65	67.25 ± 8.07	59.32 ± 7.27	110.91 ± 17.7	88.73 ± 15.26	75.52 ± 13.70	66.87 ± 17.95	72.38 ± 3.42	62.91 ± 17.46	50.85 ± 10.65	54.50 ± 14.21
AST	88.25 ± 2.61	78.75 ± 4.01	69.12 ± 3.72	71.38 ± 4.60	71.12 ± 5.18	63.75 ± 2.62	56.62 ± 2.19	40.62 ± 2.19	20.50 ± 1.38	41.50 ± 4.84	40.62 ± 2.18

m. g.: month of gestation; d. b. p.: days before parturition; w. l.: week of lactation

lambing. In our experiment, both groups showed the same changes during pregnancy, which indicates that this behaviour cannot be explained by gestation. This is in agreement with the findings of Kundu et al. (1985), who showed that whole blood and plasma Se were relatively constant during gestation. Our results, however, show an increase of Se requirements for ewes at the beginning of lactation. This higher demand was explained by Pehrson et al. (1990) who demonstrated an elevated Se elimination through the colostrum. Se does not cross the placenta in any appreciable amounts; however, that is certainly compensated for by the high Se content of the colostrum. Providing colostrum rich in Se is essential for lambs born with low levels of this mineral, as shown by the elevation of their Se concentration during the first week of life.

Table 2

Changes in the activities of whole blood gluttathione peroxidase (IU/g Hb) and serum creatine kinase and aspartate aminotranferase (IU/l) in the lambs, (expressed as mean ± SEM)

	Times				
	Birth	Week 1	Week 2	Week 3	Week 4
GSH-Px	90.13±26.55	80.46±7.76	108.56±9.04	151.54±18.30	156.86±8.42
CK	85.57±9.77	58.85±8.07	58.03±10.30	51.35±9.53	51.39±8.78
AS	50.10±4.24	47.40±1.89	17.80±3.04	23.10±1.59	33.10±1.59

With regard to GSH-Px activity during the reproductive stage, Verde et al. (1992) have shown that the activity of that enzyme decreased during gestation and especially at parturition. This is consistent with the findings of Osame et al. (1992) and Zachara et al. (1992) who have indicated that GSH-Px activity decreased significantly and was at critically low levels during the lambing and lactation periods. Our results indicate that the stage of reproduction has no significant influence on GSH-Px activity, which showed a strong decrease in the third month of pregnancy. However, gestation cannot account for that change because exactly the same phenomenon was observed in the control, non-pregnant group. Several authors (Wheatley and Beck, 1988; Andrés et al., 1994) also described similar seasonal trends in the GSH-Px activity of sheep, this tendency being especially important in regions with a low Se level.

In a selenium-deficient area, the dietary Se level limits or determines the amount of blood GSH-Px activity as antioxidant capacity. Antioxidant requirements are higher during the reproductive stage. If deficiency in Se intake occurs at a time when there is an excessive exposure to stimulators of reactive oxygen metabolites production, a relative deficiency of GSH-Px will occur, resulting in

oxidative stress. When left uncontrolled, this situation can cause extensive tissue damage, which may affect membrane permeability, enzyme function and even muscle tone (Miller et al., 1993), and the final consequence will be the animal's inability to maintain reproductive performance. During the second half of pregnancy GSH-Px activity was within the deficient range (Mañé et al., 1991; Sáez et al., 1995). In spite of this low activity, no evidence of existing pathologic conditions associated with Se deficiency was found, since the activities of the muscle markers CK and AST were within the normal range. In the same way, no distinct symptoms of nutritional myopathy were observed in the lambs, suggesting that this low Se in the ewes did not cause any alterations in the development of lambs. These findings suggest that the observed subnormal GSH-Px activity may have been compensated for by an adequate level of vitamin E in the diet. Vitamin E was not assessed in this study. However, ewes were grazing on fresh forage, considered as an adequate source of vitamin E (Maas et al., 1984; Hamliri et al., 1993), suggesting that vitamin E status was adequate. It is well known that vitamin E and Se-GSH-Px activity are the most important components of the antioxidative defence mechanisms, capable of substituting for one another. In this sense, Garce and Clarck (1991) have shown that Se-responsive diseases do not occur at the same selenium status in different regions, indicating that these differences in Se requirements may be partly explained by the varying vitamin E intakes.

Although during our study no clinical manifestations of selenium deficiency were found in the ewes and their lambs, GSH-Px activity, the measure of functional selenium in the tissues, was below the physiological levels during the second half of pregnancy. Further studies should be carried out to evaluate the influence of season and husbandry on the selenium status in sheep and to determine how much Se sheep in the region under study require for maintaining health and maximal reproductive performance.

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PLASMA PROTEINS AND HAEMATOLOGICAL PARAMETERS IN FATTENING PIGS FED DIFFERENT SOURCES OF DIETARY ZINC

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The effect of varying amounts and sources of dietary zinc on some blood parameters and the plasma protein ratios of fattening pigs was studied. During a 30-day pre-trial period, 42 pigs were depleted of Zn and divided into three groups of 14 animals each. During the 105-day experimental fattening period two experimental groups (T_{1-2}) were repleted with dietary Zn in the form of inorganic salt ($ZnSO_4$) or metallo-organic chelate (zinc methionate, ZnMET), while the control group (C) received no extra Zn. The supplements contained 84.3 and 40.9 mg Zn/kg diet, respectively. The nutritional effects of Zn were evaluated on the basis of red blood cell (RBC) count, mean corpuscular volume (MCV), haemoglobin (Hb) concentration, packed cell volume (PCV), white blood cell (WBC) and thrombocyte count, plasma total protein, albumin, and globulin concentrations, and albumin/globulin (A/G) ratio. During the parakeratosis period (days 28–56) both the basic feed (group C) and the diet enriched with 40.9 mg Zn/kg (as ZnMET) fed to group T_2 pigs resulted in a lower red blood cell (RBC) count, Hb concentration and PCV than in group T_1 fed a diet containing 84.3 mg Zn/kg (as $ZnSO_4$). Dietary Zn of organic and inorganic origin had no effect on MCV and on the WBC and thrombocyte counts of fattening pigs. At the start of the parakeratosis period (day 28), group C pigs had significantly lower, and by its end (day 56) significantly higher plasma total protein and globulin concentrations than pigs of group T_1. At the same time, no significant differences were observed in albumin concentration between group C and the two experimental groups. Throughout the trial, feed containing sufficient, and insufficient, quantities of Zn from various sources had no influence on blood plasma alpha and beta globulin concentrations. In the second part of the experiment (day 56) dietary Zn deficiency resulted in a significantly higher level of gamma globulin in group C pigs than in pigs given sufficient Zn of inorganic origin. While inorganic Zn increased some of the blood parameters, namely RBC count, Hb and possibly PCV, organic Zn seemed to be responsible for raising the proportion of plasma globulins.

Key words: Pigs, nutrition, haematological parameters, plasma proteins, zinc sulphate, zinc methionate

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A large number of research projects have dealt with the transport of zinc and its biological importance for the animal organism (cell replication, release of CO_2 from lung tissue, sexual maturation, wound healing processes, fertility and reproduction). Zinc is an essential trace element and an integral part of some 300 different enzymes (e.g., alkaline phosphatase, alcohol dehydrogenase, carboanhydrase, etc.). It has an essential role in protein synthesis as well as in the metabolism of carbohydrates and nucleic acids (Milne, 1994). It is believed that in combination with amino acids and small peptides, zinc promotes animal productivity (Herrick, 1989; Berrie et al., 1994; Rust, 1995; Sefton, 1997). Sefton (1997) claims that it is possible to direct the movement of such minerals within an organism by linking certain micro- and oligo-elements (cobalt, copper, zinc and manganese) with certain amino acids (methionine, tryptophan, lysine, histidine) and with small peptides, thus influencing the site of their deposition in selected tissues (muscle, heart, bone, spleen, liver, uterus, cellular oxygen metabolism, kidneys, hoof). We were specifically interested in the nutritive influence that Zn from various organic and inorganic sources had on the health condition of pigs (Smith et al., 1960; Mansson and Olhagen, 1967; Shanklin et al., 1968; Whitenack et al., 1978; Hartmann et al., 1995). It should be mentioned that scientists disagree among themselves as to the biological availability of Zn from organic and inorganic sources to pigs. Wedekind et al. (1994) found no differences in production results with pigs offered a diet containing zinc from either an organic or an inorganic source. However, Hill et al. (1986) stress the fact that pigs offered feed containing zinc from an inorganic source demonstrated higher rates of productivity, which accords with our recent results (Rupić et al., 1997). The controversies which still appear from data of the literature on the possible role of varying amounts and sources of dietary zinc on haematological parameters prompted the current project.

The purpose of this research was to determine the nutritive influence that varying quantities of inorganic ($ZnSO_4$) and organic (ZnMET) sources of zinc have upon the health condition of pigs. This was done by establishing the red blood cell (RBC) count and mean corpuscular volume (MCV), haemoglobin (Hb) concentration, packed cell volume (PCV), white blood cell (WBC) and thrombocyte counts, concentration of total proteins, albumin and globulin, as well as the albumin/globulin (A/G) ratio and the proportion of individual proteins in the blood plasma.

Materials and methods

A total of 42 German Landrace × Piétrain × Large White × Swedish Landrace weaned crossbreds were used in the experiment. The pigs were kept in pens, seven pigs per pen. There were seven males and seven females in each

group. All groups in the experiment comprised pigs from seven different litters, all born on the same day. Each group included an equal number of pigs from one litter (one male and one female).

Male piglets were castrated at the age of 14 days. All pigs were tattooed on the right ear with a number (from 1 to 42). All groups of animals were kept in the same microclimatic conditions. Average air temperature was 18.0 °C and relative air humidity was maintained at 78.8%. There was forced air circulation in the animal room. The pigs' health status was checked daily.

Pigs were offered the diets and drinking water *ad libitum* by means of automatic feeding and watering systems. The composition of the feed mixes is given in Table 1.

During a 30-day period preceding the experiment all piglets were acclimatised to the environment and were fed a starter diet (19.31% protein, 45.5 mg Zn/kg) but received no extra Zn supplement. This feeding regime was intended to exhaust Zn depots in the piglets. After weighing, piglets were divided into three groups: the control group (C), the first test group (T_1), and the second test group (T_2), which were uniform as to the number of animals (14 in each group) and their body mass. Average starting body mass for all groups of animals was 15.5 kg (C = 15.65 kg, T_1 = 15.41 kg, and T_2 = 15.42 kg). During the 105-day experimental fattening period all animals were fed the same basic feed (Table 1) corresponding to their body mass category. Accordingly, during the first 28 days of the experiment (up to 32 kg), all piglets were fed starter; for the next 28 days (up to 50 kg) they were fed grower, and during the final 49 days of the experiment they were fed finisher diet (average finishing body mass was 90.9 kg). This conformed to a well-established feeding regime for pigs (Allen, 1993).

Group C was given feed mix with no extra Zn added, i.e., throughout the experiment they received an average of 36.7 mg Zn/kg of diet. On average, group T_1 was supplemented with an extra 84.3 mg Zn/kg feed (starter: 82.5, grower: 86.3, finisher: 84.2 mg Zn/kg diet), from $ZnSO_4$. Group T_2, on the other hand, was given an extra Zn from ZnMET with an average amount of 40.9 mg Zn/kg feed (starter: 56.3, grower: 42.3, finisher: 31.3 mg/kg diet). As sources of Zn, $ZnSO_4 \times H_2O$ (B. B. V. Chemie, Duisburg, Germany), and Zn-methionate (PLIVA, Zagreb, Croatia) were used. Zinc compounds were mixed into vitamin-mineral premixes which were added to the starter diet at a concentration of 1%, and to the grower and finisher diets at a concentration of 0.5%. The vitamin-mineral premixes were also the products of PLIVA.

Table 1

Composition of feed mixes

Ingredient (%)	Starter	Grower	Finisher
Maize	46.05	42.50	39.50
Barley meal	22.00	33.45	41.00
Wheat middlings	–	–	3.50
Soybean meal	19.00	13.00	9.50
Sunflower meal	–	6.00	–
Lucerne meal	1.50	2.50	3.40
Fodder yeast	6.67	–	1.00
Fat	1.20	–	–
Iodised salt	0.30	0.35	0.50
Limestone	1.15	1.00	0.80
Dicalcium phosphate	1.00	0.70	0.30
DL-methionine	0.13	–	–
Vitamin-mineral premix SP*	1.00	–	–
Vitamin-mineral premix ST*	–	0.50	0.50
Total	100.00	100.00	100.00
Composition by analysis**			
Dry matter (%)	88.56	89.20	88.80
Crude protein (%)	19.31	15.68	13.30
Crude fat (%)	3.75	3.00	2.60
Crude fibre (%)	3.77	4.81	4.02
Ca (%)	0.78	0.72	0.60
P (%)	0.65	0.53	0.38
Mn (mg/kg)	46.00	48.70	52.37
Fe (mg/kg)	315.00	178.26	139.40
Cu (mg/kg)	32.50	20.24	12.30
Zn (mg/kg) C	45.50	38.00	31.00
Zn (mg/kg)† T_1	128.00	124.26	115.20
Zn (mg/kg)¶ T_2	101.80	80.30	62.33

*Vitamin-mineral premixes contained:

Zn-free VMP–SP provided the following per kilogram: 1,000,000 IU vitamin A; 100,000 IU vitamin D_3; 1,500 mg vitamin E; 200 mg vitamin K_3; 200 mg vitamin B_1; 400 mg vitamin B_2; 2,500 mg niacin; 1,500 mg *D*-pantothenic acid; 300 mg vitamin B_6; 2 mg vitamin B_{12}; 10 mg biotin; 50,000 mg choline chloride; 10,000 mg Fe; 2,000 mg Cu; 3,000 mg Mn; 75 mg I; 30 mg Co; 10 mg Se. Additions of Zn were 8,000 mg from $ZnSO_4$ and 4,000 mg from ZnMET per kilogram.

Table 1 continued

Zn-free VMP–ST provided the following per kilogram: 1,000,000 IU vitamin A; 100,000 IU vitamin D_3; 2,400 mg vitamin E; 400 mg vitamin K_3; 400 mg vitamin B_1; 600 mg vitamin B_2; 3,000 mg niacin; 2,000 mg *D*-pantothenic acid; 400 mg vitamin B_6; 3 mg vitamin B_{12}; 100,000 mg choline chloride; 16,000 mg Fe; 4,000 mg Cu; 8,000 mg Mn; 150 mg I; 40 mg Co; 20 mg Se. Additions of Zn were 16,000 mg from $ZnSO_4$ and 8,000 mg from ZnMET per kilogram.

**Official methods were used throughout (A.O.A.C., 1984); †After addition of $ZnSO_4$ to vitamin-mineral premix; ¶After addition of ZnMET to vitamin-mineral premix

Concentration of Zn in blood serum was determined by flame atomic-absorption spectrometry, under standard instrumental conditions (Perkin-Elmer 305B atomic absorption spectrophotometer, Perkin-Elmer, Norwalk, CT, USA). Measuring samples were prepared by fivefold dilution of original sera with doubly purified water and measured versus standard solutions prepared in 5% glycerol (Perkin-Elmer, 1976; Butrimovitz and Purdy, 1977; Smith et al., 1979).

For haematological and biochemical analyses, blood samples were taken using EDTA four times during the experiment, on days 1, 28, 56 and 105. Blood samples were taken from the brachial region (by puncture of the vena cava cranialis) at the same time of the day (8–11 a.m.) from the same animals and in the same group sequence. Blood was sampled from each individual in the group after starvation for 12 h. Five hours later the following haematological and biochemical parameters were determined: RBC count, MCV, Hb concentration, PCV, thrombocyte count, WBC count, total proteins, albumins, globulins, A/G ratio, and the proportions of plasma proteins.

All haematological parameters were determined using the SYSMEX SF 3000 (Japan) apparatus and the corresponding kits of reagents. Total proteins were defined using the standard biuret-method of Wolfson et al. (1948) with Weichselbaum's reagent (1946), while albumins and globulins were determined by electrophoresis. Semimicroelectrophoretic separation of proteins in plasma was performed on a cellogel base under the voltage of 240 V for 30 min using the Chemetron Milan (Italy) apparatus. After separation, protein fractions were detected with Ponceau S dye, cut, eluted with 80% acetic acid and the eluates measured spectrometrically at 520 nm.

Animals used in this study were maintained in facilities approved by the Croatian Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the Croatian Ministry of Agriculture.

All statistical analyses were performed using the SAS GLM procedure (SAS Institute, 1989).

Results

Status of animals

When examined after the 30-day pre-trial period, none of the pig groups showed parakeratotic changes or disturbances of the general health condition.

After day 28 of the experiment it was observed that pigs of group C were not eating well, they manifested intense pruritus and their skin, particularly in the lower region of the abdomen, became reddened; they also exhibited round-shaped, scabbed swellings of about two cm in diameter which tended to merge. These symptoms disappeared at about day 77 of the experimental fattening. From the clinical aspect this was a typical case of parakeratosis resulting from Zn deficiency of the diet. Pigs of the two test groups (T_1 and T_2) manifested no changes of their skin, nor did their food intake decrease.

This clinical picture was confirmed by the concentration of Zn in the blood serum (Fig. 1, Rupić et al., 1997). The transient increase of serum Zn in group C, which was observed on day 28, was followed by a severe and steady decrease to the hypophysiological values. Contrary to this, serum Zn concentration in groups T_1 and T_2 remained steadily at the upper limit of the physiological range. The strong linear correlation between serum zinc level and alkaline phosphatase activity with a coefficient of correlation of 0.90 additionally confirmed the control pigs' hypozincaemic status.

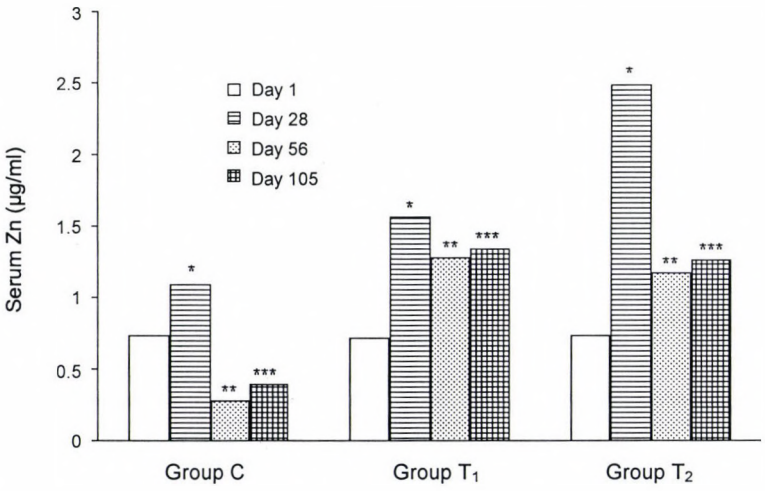

Fig. 1. Average serum zinc concentrations during experimental fattening (n = 14). Significant difference ($P < 0.05$) of average Zn levels between either of the two trial groups and the control group at days 28 (*), 56 (**) and 105 (***)

Haematological parameters

Haematological parameters of all groups, as found during the experiment, are shown in Table 2.

Table 2

Haematological parameters in pigs throughout the experimental period (n = 14)

	Day	Group		
		C (mean ± SD)	T_1 (mean ± SD)	T_2 (mean ± SD)
RBC (10^{12}/L)	1	5.87 ± 0.69	6.16 ± 0.55	6.11 ± 0.59
	28	6.45 ± 0.45	¶6.79 ± 0.48	¶6.33 ± 0.37
	56	6.91 ± 0.38	¶7.20 ± 0.41	¶6.74 ± 0.44
	105	6.72 ± 0.64	6.80 ± 0.48	6.98 ± 0.47
MCV (f/L)	1	57.14 ± 2.48	57.57 ± 4.36	56.43 ± 2.17
	28	56.71 ± 2.02	55.86 ± 2.35	55.36 ± 2.34
	56	†55.79 ± 2.12	57.36 ± 2.56	†58.43 ± 3.57
	105	58.29 ± 2.09	57.86 ± 2.51	59.00 ± 2.88
Haemoglobin (g/L)	1	110.00 ± 9.81	110.79 ± 4.85	107.50 ± 11.63
	28	111.29 ± 5.55	¶115.50 ± 7.96	¶107.36 ± 9.62
	56	†120.29 ± 4.56	†;¶126.14 ± 9.01	¶119.00 ± 9.14
	105	123.29 ± 11.80	124.36 ±10.94	129.50 ± 7.25
PCV (L/L)	1	†0.36 ± 0.04	†;¶0.39 ± 0.02	¶0.36 ± 0.03
	28	0.37 ± 0.02	¶0.38 ± 0.02	¶0.36 ± 0.03
	56	†0.39 ± 0.02	†0.42 ± 0.03	0.40 ± 0.04
	105	†0.40 ± 0.03	¶0.40 ± 0.03	†;¶0.42 ± 0.02
WBC (10^9/L)	1	21.04 ± 2.32	20.99 ± 3.32	20.99 ± 4.53
	28	20.05 ± 2.94	20.32 ± 3.30	20.29 ± 3.94
	56	24.87 ± 2.81	24.01 ± 5.18	22.54 ± 4.30
	105	19.33 ± 3.14	20.36 ± 2.76	20.04 ± 3.73
Thrombocytes (10^9/L)	1	282.93 ± 71.14	270.29 ± 91.43	271.79 ± 76.60
	28	308.71 ± 94.56	326.64 ± 99.61	322.79 ± 94.00
	56	214.71 ± 97.11	233.21 ± 102.93	240.21 ± 63.67
	105	169.07 ± 73.32	222.21 ± 73.71	189.71 ± 63.16

†Significant difference between either of the test groups and group C ($P < 0.05$); ¶Significant difference between test groups ($P < 0.05$)

No significant differences were found in RBC count, either at the start (day 1) or at the end (day 105) of the trial, between group C and the test groups. However, at day 28 and day 56 pigs in group T_1 showed a significantly higher RBC count than those in group T_2 ($P < 0.05$).

At the start of the experiment, on day 28, and at the end of the experiment, MCV was not significantly different in the different groups of fattening pigs, although on days 28 and 56 pigs in group T_1 had significantly higher ($P < 0.05$) Hb concentration than those in both group C and group T_2.

On the first day of the experiment, pigs in group T_1 showed a significantly higher ($P < 0.05$) PCV than those in groups C and T_2. By day 28 their PCV ($P < 0.05$) exceeded only that of group T_2 pigs, while on day 56 they showed a PCV significantly higher than the pigs of group C.

Throughout the experiment, no significant differences were seen in WBC and thrombocyte counts among the groups.

Plasma proteins

Table 3 shows the concentrations of total proteins, albumin, globulin and the A/G ratio in the blood plasma of pigs during the experiment.

At the start and at the end of the experiment no significant differences were found in the concentration of total plasma proteins among the groups. On day 28, group T_1 pigs showed a significantly higher ($P < 0.05$) concentration of total proteins than the controls, while on day 56 their total protein concentration was significantly lower ($P < 0.05$) than in groups C and T_2.

At the start of the experiment, pigs of group C showed a significantly higher ($P < 0.05$) plasma albumin concentration than those in either of the test groups. On day 28, group T_1 pigs showed a significantly higher, and on day 56 a significantly lower ($P < 0.05$), plasma albumin concentration than group T_2 pigs.

On days 1 and 28 of the experiment, group C pigs showed a significantly lower ($P < 0.05$) plasma globulin concentration than those in either of the test groups; on day 56, however, group T_1 pigs were found to have a significantly lower ($P < 0.05$) globulin concentration than those in group C and T_2.

At the start of the experiment, group C pigs showed a significantly higher A/G ratio than those in either of the test groups. On day 28, group C pigs and those in group T_1 showed a significantly higher ($P < 0.05$) A/G ratio than those in group T_2, while in the second half of the experimental period (days 56 and 105) no significant differences were observed in the A/G ratio among fattening pigs in any of the three groups.

Table 3

Protein concentration in the plasma of pigs throughout the experimental period (n = 14)

	Day	Group		
		C (mean ± SD)	T_1 (mean ± SD)	T_2 (mean ± SD)
Total proteins (g/L)	1	61.14 ± 2.77	61.00 ± 3.11	61.36 ± 2.31
	28	†78.29 ± 8.35	†84.29 ± 6.38	80.50 ± 7.89
	56	†76.50 ± 9.24	†,¶68.21 ± 6.07	¶79.21 ± 3.60
	105	85.36 ± 4.72	83.14 ± 3.08	86.00 ± 5.80
Albumin (g/L)	1	†35.23 ± 4.06	†29.97 ± 3.37	†30.28 ± 5.53
	28	38.79 ± 6.01	¶41.70 ± 3.97	¶35.89 ± 4.41
	56	33.66 ± 6.65	¶32.66 ± 3.34	¶35.70 ± 3.07
	105	45.85 ± 5.04	46.26 ± 4.15	44.96 ± 8.41
Globulin (g/L)	1	†25.20 ± 4.20	†30.96 ± 4.47	†30.94 ± 4.70
	28	†39.51 ± 3.99	†42.66 ± 3.62	†44.61 ± 6.46
	56	†42.91 ± 6.42	†,¶35.55 ± 6.17	¶43.73 ± 4.32
	105	39.86 ± 7.71	36.52 ± 3.21	41.07 ± 7.43
A/G ratio	1	†1.38 ± 0.42	†0.98 ± 0.22	†1.01 ± 0.40
	28	†0.99 ± 0.14	¶0.97 ± 0.09	†,¶0.80 ± 0.15
	56	0.79 ± 0.18	0.94 ± 0.23	0.81 ± 0.14
	105	1.20 ± 0.35	1.25 ± 0.21	1.13 ± 0.32

†Significant difference between either of the test groups and group C ($P < 0.05$); ¶Significant difference between the test groups ($P < 0.05$)

The proportions of individual plasma proteins as found during the experiment are shown in Table 4.

Throughout the experimental period no significant differences were observed in the proportion of alpha globulin ($\alpha_1 + \alpha_2$) between groups of pigs used in the experiment. Similarly, no significant differences were observed in the proportion of beta globulin between groups of fattening pigs at the start, and on days 56 and 105, of the experiment; however, on day 56 group C pigs showed a significantly higher ($P < 0.05$) proportion of gamma globulin than those in group T_1.

Table 4

Proportion of plasma proteins in pigs during the experimental period (n = 14)

	Day	Group		
		C (mean ± SD)	T_1 (mean ± SD)	T_2 (mean ± SD)
Albumin (%)	1	†57.36 ± 7.50	†48.89 ± 5.86	†48.95 ± 7.85
	28	†49.21 ± 4.07	¶48.85 ± 2.83	†,¶44.40 ± 4.44
	56	44.21 ± 7.06	47.87 ± 5.70	44.69 ± 4.32
	105	53.68 ± 7.06	55.35 ± 3.98	51.95 ± 8.50
Alpha globulin ($\alpha_1 + \alpha_2$, %)	1	21.14 ± 3.53	23.14 ± 1.71	23.39 ± 3.14
	28	19.64 ± 4.19	19.71 ± 1.23	19.62 ± 3.02
	56	19.22 ± 2.66	20.06 ± 2.71	19.49 ± 3.17
	105	15.72 ± 1.65	15.43 ± 2.09	17.20 ± 4.38
Beta globulin (%)	1	5.70 ± 2.36	6.59 ± 2.70	6.19 ± 1.95
	28	5.66 ± 3.25	¶4.51 ± 1.36	¶8.13 ± 5.37
	56	5.38 ± 1.80	4.87 ± 1.75	5.66 ± 2.10
	105	3.99 ± 1.45	4.58 ± 1.84	5.02 ± 1.84
Gamma globulin (%)	1	†15.70 ± 3.86	†21.38 ± 5.13	†21.48 ± 6.01
	28	24.35 ± 3.98	26.12 ± 2.32	27.84 ± 4.92
	56	†31.91 ± 4.46	†27.19 ± 3.39	30.14 ± 4.39
	105	26.61 ± 6.56	24.64 ± 3.85	24.84 ± 3.64

†Significant difference between either of the test groups and group C ($P < 0.05$); ¶Significant difference between the test groups ($P < 0.05$)

Discussion

Pigs of the control group were offered feed mixtures containing no added Zn, and from day 25 until the end of the experiment (day 105) all animals in the group manifested restlessness and pruritus; in the period from day 27 to day 77 parakeratotic changes were observed on the animals' skin. From day 77 until the end of the experiment, those changes gradually disappeared. Neither such changes nor signs of pruritus and restlessness were observed during the experimental period in group T_1 pigs whose feed contained 84.3 mg Zn/kg of an inorganic source ($ZnSO_4$), nor in group T_2 pigs, whose diet contained 40.9 mg Zn/kg of an organic source (ZnMET). Both test groups attained a significantly higher growth rate than group C: group T_1 by 18.8% and group T_2 by 20.9% (Rupić et

al., 1997). Additionally, the same research demonstrated that pigs in both test groups achieved better feed conversion than those in group C: group T_1 by 3.7% and group T_2 by 2.7%.

Haematological parameters

Almost similar value profiles were observed during the experimental period in all test groups for each investigated haematological parameter.

A remarkable rise in the values of RBC count, Hb and PCV was recorded for all groups by day 56 of the experiment, when values stabilised. In the same period the highest RBC count was observed in group T_1, i.e. on days 28 and 56 it was significantly higher than in group T_2. No significant difference was observed in RBC count between group C and groups T_1 and T_2. The results indicate that RBC count and Hb concentration increased rapidly in pigs whose feed contained Zn from an inorganic source, whereas this was not the case in pigs offered a diet containing Zn from an organic source. An analogy of trends and comparative values for RBC count, Hb and PCV in groups C and T_2 point to the conclusion that parakeratosis cannot be regarded as a dominant, or the sole, factor which determines the values of haematological parameters. It is the inorganic source of Zn ($ZnSO_4$) in the feed which appears to be responsible for the increase in the values of these haematological parameters. Results for RBC count, Hb concentration and PCV in pigs of group T_2 show that the organic Zn (ZnMET) contained in the diet had no influence either on erythropoiesis or on haemoglobin synthesis in erythrocytes during the experiment, which was not the case when pigs were offered a feed containing Zn from an inorganic source ($ZnSO_4$).

MCV values showed practically no significant differences, which indicates that the quantity and source of Zn, whether it be sufficient or insufficient, had no influence on MCV in pigs during the experiment.

The Hb concentrations measured accord fully with the RBC count. During the period of parakeratotic changes on skin (days 28 and 56), significantly higher values were observed in group T_1 than in groups C and T_2. Quantities of Zn from an inorganic source in the diet had a positive influence on Hb concentration of the blood as compared to applied quantities of Zn from an organic source.

On the basis of the foregoing it could therefore be concluded that during the clinical manifestation of parakeratosis in pigs offered a feed containing no added Zn (group C), erythropoiesis and Hb synthesis in erythrocytes were lower than in pigs fed a diet containing a sufficient quantity of Zn from an inorganic source ($ZnSO_4$). It should be mentioned that the RBC count, the Hb level and the PCV of pigs fed the diet containing Zn from an organic source (ZnMET) were similar to the haematological parameters in pigs offered a feed with no Zn

added, but were significantly lower in relation to those of pigs offered a diet with Zn from an inorganic source during the period of rapid growth and formation of body bio-mass (days 28 and 56 of the experiment). These findings are in harmony with the role that Zn normally plays in the biochemical processes of the organism (cell proliferation, immune functions of cells, and others: Sandstead, 1995). However, the value increases observed in group T_1 were sudden and transient. At the same time, values obtained in group T_2 indicate a trend of slight but steady growth which, over the 105-day experimental period, still does not significantly exceed those in groups C and T_1 except for PCV. It would therefore be considered advantageous to conduct a long-term experiment, the results of which could provide a definite answer regarding the influence of organic zinc on haematological parameters.

Findings identical with our own results with regard to RBC count, Hb concentration and PCV were obtained by Hartmann et al. (1995) in their dietary experiment where groups of pigs were offered feeds containing sufficient and insufficient quantities of Zn.

Bearing in mind the fact that during the experiment no significant differences were observed in WBC or thrombocyte counts among the groups of pigs, it can be concluded that additions of Zn from various sources had no significant influence on leukocytopoiesis and thrombocytopoiesis in fattening pigs.

Haematological parameters (RBC count, MCV, Hb concentration, PCV, WBC and thrombocyte counts) obtained in our experiment in all groups of pigs (C, T_1, T_2) coincide with the values given as normal for pigs by Imlah and McTaggart (1977), Scheunert and Trautmann (1987), and Odink et al. (1990).

Plasma proteins

The trends of changes in the value of plasma proteins were analogous in all three groups of pigs involved in the experiment.

Significant differences in the concentration of plasma proteins between group C and test group T_1 were observed only during the period of pathological skin changes (parakeratosis on days 28 and 56 of the experiment). On day 28, pigs in group C offered a feed containing no added zinc had a significantly lower concentration, but on day 56 of fattening they had a significantly higher concentration of total proteins than pigs in group T_1, whose diet contained sufficient quantities of inorganic zinc. During the experiment no significant differences in total plasma protein concentration were observed between pigs of group C and those of group T_2. Both the increase of total protein concentration at the onset of the parakeratosis period and its decrease in the second part of that period were less pronounced in group C than in the test groups. Our results on the concentration of plasma proteins in group C pigs during the period of parakeratotic skin

changes partially conform to the findings of Hartmann et al. (1995) while they are fully consistent with those of Smith et al. (1960).

Only at the start of the experiment did pigs of group C show a significantly higher concentration of plasma albumin than those of both test groups, while no significant differences were observed during the remaining part of the experiment. The significant differences observed in the concentration of plasma albumin on the first day in the pigs used in the experiment can be attributed to the genetic influence of the breeds used for obtaining crossbred pigs for test fattening.

Up to day 28 of the experiment, pigs in group C had a significantly lower level of plasma globulin than those in either of the test groups, while on day 56 group C pigs had a significantly higher level of plasma globulin than pigs in group T_1. This data indicates that a diet lacking in Zn leads to an equalised or even increased concentration of plasma globulin in relation to pigs in the experimental groups whose feed contained sufficient quantities of inorganic and organic Zn. Our findings regarding plasma globulin concentrations in groups of pigs offered a feed containing sufficient and insufficient quantities of Zn are at variance with results obtained by Smith et al. (1960). It was also found that, as with the phenomenon observed for RBC count, Hb concentration and PCV, plasma globulin concentration underwent a sudden and remarkable, although transient, rise in all experimental groups.

The A/G ratio is a result of the previously presented concentrations of albumins and globulins. Between days 28 and 56 the A/G ratio was minimal in all three test groups, but at the end of the experiment it showed a distinct increase. A significantly higher A/G ratio in group C in relation to groups T_1 and T_2 was observed only at the start of the experiment, and for the experimental period as a whole the values equalised between the groups. This means that the A/G ratio cannot be correlated with the appearance of parakeratosis. These results are at variance with the findings of the A/G experiment conducted by Smith et al. (1960), who found more albumin than globulin in the plasma of healthy pigs, while in pigs affected by parakeratosis the level of globulin exceeded that of albumin.

Our values of total plasma proteins, albumins and globulins for pigs in all three groups (C, T_1 and T_2) fall within the normal range given for pigs by Scheunert and Trautmann (1987) and Forenbacher (1993), but are slightly higher than the values presented by Imlah and McTaggart (1977) and Odink et al. (1990) for healthy pigs.

Throughout the entire period of the experiment no significant differences were noticed in the proportion of plasma alpha globulins between pigs offered feed with insufficient (group C) and sufficient but varying quantities and sources

of Zn. It can be said that in the parakeratosis period (days 28 and 56) no disorders in the synthesis of plasma alpha globulin were observed in group C.

No significant differences in beta globulin concentration were found between group C and the test groups during the trial, indicating that beta globulins were not affected by the deficiency of zinc.

At the start of the experiment, group C pigs had a significantly lower proportion of plasma gamma globulins than pigs in either of the test groups. On day 56 they had the highest proportion of gamma globulin in the plasma, significantly higher than group T_1 pigs. It is clear that at the beginning of the parakeratosis period the proportion of gamma globulin in the plasma is somewhat lower, whereas at the end of that period it is higher in the control pigs than in pigs of either test group. This would indicate that during the feeding of a zinc-deficient diet, or rather during an extended period of clinically visible parakeratosis, the proportion of plasma gamma globulin increases, i.e., the level of antibodies in the circulation rises.

Our values obtained for the proportion of albumins, alpha globulins and gamma globulins in the plasma of pigs in all groups exceed the values presented as normal by Scheunert and Trautmann (1987), and are the same as those presented by Forenbacher (1993). However, our findings on the proportion of beta globulins are remarkably lower than the values reported by Scheunert and Trautmann (1987) and Forenbacher (1993). All proportions of the given fractions of plasma proteins in pigs of all test groups in our experiment are higher than the proportion of plasma proteins found in pigs by Smith et al. (1960).

Zinc is known to have a higher absorption rate from its complexes with amino acids such as methionine, lysine and small peptides (Herrick, 1989; Wedekind and Baker, 1990; Wedekind et al., 1992; Wedekind et al., 1994; Hemken, 1997; Sefton, 1997; Rupić et al., 1997) than from inorganic compounds. We have found that the bioavailability of Zn from ZnMET is twice as high as that from $ZnSO_4$, which is documented by comparable serum Zn values in both test groups, T_1 and T_2 (Fig. 1).

In conclusion it can be stated that haematological parameters and plasma proteins did not follow the pattern of serum Zn levels during the experiment. This was clearly demonstrated by the fact that significant differences in serum Zn concentration between group C and both of the trial groups, T_1 and T_2, were not reflected in analogous trends of the haematological parameters and the plasma proteins. This means that these values cannot be used as indicators of either the Zn status of pigs or the bioavailability of dietary zinc. This makes the significant differences observed in RBC count and Hb concentration between groups T_1 and T_2 even more valuable. They might be attributed to supplementation with inorganic dietary zinc ($ZnSO_4$). At the same time, the influence of zinc from an organic source (ZnMET) is manifested only in a somewhat increased

concentration of plasma globulins. These facts could be explained by the different metabolic routes of Zn from inorganic and organic sources in the body, or rather by its dissimilar distribution and deposition in certain specific depots (liver, bones, hair, metallothioneine). A possibly more effective deposition of inorganic Zn in hair and bones, as indicated by our recently obtained results (Rupić et al., 1997) could be associated with a more pronounced haematopoiesis.

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DOLPHIN MORBILLIVIRAL INFECTION FROM THE MEDITERRANEAN SEA DID NOT SPREAD INTO THE ADRIATIC SEA

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In July of 1990, a mass mortality of striped dolphins due to morbillivirus infection had begun in the western Mediterranean. By 1992, the infection had spread to the eastern Mediterranean and the Aegean Sea. Other dolphin species in the Mediterranean were not found to have died due to this infection, although it is possible for many species of marine mammals to be infected. In 1994, it was published that morbillivirus infection had caused Atlantic bottlenose dolphin mortality in the USA. Although striped dolphins are not residents of the Adriatic Sea, it was hypothesised that the infection could have spread from them to Adriatic bottlenose dolphins. From October 1990 through April 1997, 16 dolphin carcasses found along the Croatian Adriatic coast were examined. Tissues were examined by light microscopy for syncytia and inclusion bodies, histopathologic lesions characteristic of dolphin morbillivirus infection, and by detection of morbilliviral RNA by a reverse transcriptase polymerase chain reaction (RT-PCR). No signs of morbillivirus infection were found in the examined animals. It was concluded that this infection had not spread to dolphins of the Adriatic Sea up until that date.

Key words: Bottlenose dolphin (*Tursiops truncatus)*, morbillivirus, epizootic, survey, Adriatic Sea, Croatia

At the present time, the bottlenose dolphin, *Tursiops truncatus* (Montagu, 1821) is the only dolphin species resident in the Adriatic Sea (Gomerčić et al., 1994; Notarbartolo di Sciara et al., 1994). At the same time, the most abundant dolphin species in the Mediterranean Sea has been the striped dolphin, *Stenella coeruleoalba* (Aguilar and Raga, 1993), which is not resident in the Adriatic. Only one striped dolphin has been found in the Croatian Adriatic Sea (Gomerčić et al., 1994) and it was included in this study. Another marine mammal species

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in the Adriatic Sea is a very small population of Mediterranean monk seal, *Monachus monachus* (Gomercic et al., 1984; Antica et al., 1994).

Morbilliviruses are extremely contagious, causing measles in humans, rinderpest in bovines, canine distemper and "peste des petits ruminants" in goats and sheep. Phocid distemper virus, dolphin morbillivirus and porpoise morbillivirus cause infections in marine mammals (Haas and Barrett, 1996). Modified morbilliviruses — phocid distemper virus-2 (probably a strain of, or identical with, canine distemper virus) and phocid distemper virus-1 (Visser et al., 1993), which caused an infection among Baikal seals, *Phoca sibirica* (Grachev et al., 1989; Cvetnić, 1991; Visser et al., 1993) and harbour seals, *Phoca vitulina* (Kennedy et al., 1988; Cvetnić, 1991; Visser et al., 1993) — were recently detected. Many seals have died in Baikal Lake and in Northern Europe due to being infected by that virus. Morbillivirus infection in Northern Europe has also been detected in harbour porpoises, *Phocoena phocoena* (Kennedy et al., 1991; Kennedy et al., 1992). Van Bressem et al. (1993) detected morbillivirus neutralising serum antibodies in a common dolphin (*Delphinus delphis*) beached on the Sardinian coast (Cagliari) in Italy in July 1990, and Di Guardo et al. (1995) in an adult minke whale (*Balaenoptera acutorostrata*) beached on the Tyrrhenian coast of Tuscany in Italy in 1993. In July 1990, mass deaths of striped dolphins began in the western Mediterranean due to morbillivirus infection (Domingo et al., 1990; Van Bressem et al., 1991; Gomerčić and Huber, 1991; Di Guardo et al., 1992; Aguilar and Raga, 1993; Visser et al., 1993). Morbillivirus infection in Mediterranean striped dolphins began spreading eastwards, and by 1991 it had spread to the central Mediterranean, and by 1992 to the eastern Mediterranean and the Aegean Sea (Aguilar and Raga, 1993; Cebrian, 1995), where it caused the death of numerous striped dolphins. By July 1991 the infection had spread to the whole of the Ionian Sea (Cebrian, 1995). In the Mediterranean Sea, morbillivirus infection did not spread to other dolphin species and there are no data relating to its spread to Mediterranean monk seals (Cebrian, 1995)*. As late as the end of 1994 it was published that morbillivirus infection was proved to have spread to Atlantic bottlenose dolphins (*Tursiops truncatus*) on the Atlantic coast of the USA in 1987/88 (Lipscomb et al., 1994*a*). In 1993 and 1994, morbillivirus infection was diagnosed in dead bottlenose dolphins in the Gulf of Mexico (Lipscomb et al., 1994*b*, 1996). In 1995, the infection was discovered among long-finned (*Globicephala melas*) and short-finned (*Globicephala macrorhyn-*

* In the summer of 1997 about half the population of this highly endangered species, which inhabits the Mauritanian coast, was reported to have been killed by morbillivirus [Osterhaus, A., Groen, J., Niesters, H., van de Bildt, M., Martina, B., Vedder, L., Vos, J., van Egmond, H., Sidi, B. A. and Barham, M. E. O. (1997): Morbillivirus in monk seal mass mortality. Nature **388,** 838–839.].

chus) pilot whales, and even among Florida manatees, *Trichechus manatus latirostris* (Duignan et al., 1995*a*, 1995*b*).

The most significant histologic evidence of dolphin morbilliviral infection are multinucleated syncytia of lung epithelial cells and intranuclear and intracytoplasmic inclusion bodies in cells, particularly of lymph nodes, although morbilliviruses very often also affect an animal's brain (Domingo et al., 1990).

Although striped dolphins are not resident in the Adriatic Sea, it was feared that the infection could spread from them to Adriatic bottlenose dolphins (*Tursiops truncatus*), and even to Mediterranean monk seals (*Monachus monachus*).

Materials and methods

From October 1990 to April 1997, 15 bottlenose dolphins, *Tursiops truncatus* (Montagu, 1821) and 1 striped dolphin, *Stenella coeruleoalba* (Meyen, 1833) found dead in the Croatian part of the Adriatic Sea (between about 42°15'N and 45°35'N latitude) were examined. The dolphins were measured according to Perrin (1975) and aged by growth-layer groups in the dentine according to Slooten (1991). All carcasses were autopsied. Samples of lung, bronchial, mediastinal and parapharyngeal lymph nodes, pharyngeal mucous membrane, livers, kidneys, spleens, gonads, brains and muscles, stained by haematoxylin and eosin, were studied by light microscopy. Eighteen 10% formalin-fixed samples of lungs, bronchial lymph nodes, parapharyngeal lymph node, pharyngeal mucous membrane, livers, kidneys and muscles from 7 animals (Table 1) were examined for presence of morbillivirus, using the reverse transcriptase polymerase chain reaction (RT-PCR) technique (Krafft et al., 1995), at the Department of Veterinary Pathology, and the Department of Cellular Pathology, Armed Forces Institute of Pathology, Washington DC, USA. The dolphins which were partly autolysed were primarily selected for RT-PCR analyses. The RT-PCR was performed using the following primers: the first primer used for reverse transcription was 5'-ATTGGGTTGCACCACTTGTC-3', and the second added for PCR was 5'-ATTAAAAAGGG(GC)ACAGGAGAGAGATCAGCC-3'. The primers were designed to amplify the conserved region of the morbillivirus phosphoprotein gene. As a control for amplifiable RNA, RT-PCR for beta-actin was performed in each case.

Table 1

Dolphins (*Tursiops truncatus* and *Stenella coeruleoalba*) found dead in the Croatian part of the Adriatic Sea and examined for morbillivirus infection

Species	Found month/year	Sex	Age year	Location north/east	Body length cm	Body mass kg	Morbilliviral RNA molecules	Morbilliviral syncytia or inclusion bodies
T. truncatus	10/90	F	4	45°03'N,13°35'E	250	204	N[2,3]	N
T. truncatus	11/90	F	< 1	45°05'40"N,13°38'40"E	164	52	N[4,5]	N
S. coeruleoalba	10/91	?	adult	42°55'N,17°11'25"E	?	?	–	–
T. truncatus	6/92	M	7	45°04'N,13°34'E	263	240	N[2,*]	N
T. truncatus	summer/93	?	?	44°23'30"N,14°40'E	?	?	–	N
T. truncatus	9/93	M	3–4?	44°33'40"N,14°23'E	242	?	N[1,2,3]	N
T. truncatus	3/94	F	6	45°04'25"N,13°38'20"E	219	120	N[1,2,6]	–
T. truncatus	5/94	F	6	44°52'05"N,14°E	265	?	N[1,2,6]	–
T. truncatus	5/94	F	fetus	44°52'05"N,14°E	107	9,1	N[1,2,3,7]	–
T. truncatus	9/94	M	< 1	42°43'45"N,16°53'40"E	163	75	–	N
T. truncatus	4/95	F	adult	45°18'N,14°33'E	?	?	–	N
T. truncatus	12/95	M	9	43°43'40"N,15°53'30"E	278	237	–	N
T. truncatus	4/96	F	5	44°49'30"N,13°51'05"E	240	?	–	N
T. truncatus	8/96	?	young	43°12'30"N,16°33'20"E	210	?	–	N
T. truncatus	10/96	M	12	45°06'05"N,13°37'50"E	290	279	–	N
T. truncatus	4/97	F	7	44°46'20"N,14°19'15"E	?	?	–	N

F = female; M = male; N = negative; – = not examined; RT-PCR examined organs and tissues: [1] = liver; [2] = kidney; [3] = lungs; [4] = parapharyngeal lymph node; [5] = mucous membrane of pharynx; [6] = muscle; [7] = bronchial lymph node; * = result uncertain

Results

The only striped dolphin, stranded in October 1991 at Lumbarda beach on Korčula Island, was a physically mature animal and, according to interviews with witnesses, it had displayed some neurological symptoms, i.e., before stranding the animal rolled and swam in small circles. Neither histological nor RT-PCR-technique study of this striped dolphin was done as there was no soft tissue remaining of this animal by the time the remains were reached by us.

The examined dolphins were of both sexes (8 female, 5 male, 3 undetermined), aged from preterm fetus and cub (< 1 year old) to 12-year-old animals, a body mass of between 9 and 279 kg, and a total body length of between 107 and 290 cm (Table 1).

Autopsies and histological studies revealed the following changes in dead dolphins from the Croatian part of the Adriatic Sea: pneumonia, chronic fibrinous bilateral arthritis (*Aeromonas* sp.) of shoulder joints, spondylosis, moderate to strong diffuse fatty changes in liver with extensive subcutaneous (between blubber and muscles) oedema and moderate hydrops of body cavities, splenic atrophy, mild multifocal subacute pharyngitis with superficial fungal hyphae, moderate multifocal acute pneumonia with gram-negative bacilli, moderate multifocal granulomatous and eosinophilic pneumonia, minimal multifocal interstitial lymphoplasmacytic nephritis, and nematodes in the lungs, intestine and urethra.

Light-microscopic histological examination of the lungs, bronchial, mediastinal and parapharyngeal lymph nodes, pharyngeal mucous membrane, livers, kidneys, spleens, gonads, brains and muscles of all unautolysed bottlenose dolphins revealed not a single instance of morbilliviral infection. Neither syncytia nor inclusion bodies were found in any of those organs of twelve dead bottlenose dolphins taken from the Adriatic Sea.

All examined specimens of lungs, bronchial lymph nodes, parapharyngeal lymph nodes, pharyngeal mucous membrane, livers, kidneys and muscles of all seven studied bottlenose dolphins were negative for morbillivirus by the RT-PCR technique.

Discussion

The presented findings suggest that dolphin morbillivirus infection has not spread throughout the Croatian part — nor, very probably, throughout the whole — of the Adriatic Sea up until this moment in time. This conclusion is supported by Di Guardo et al. (1995) who wrote: "Despite the presence of lesions characteristic of morbilliviral pneumonia in two other striped dolphins stranded on the coast of Apulia in 1991, no morbillivirus antigen was detected in the tissues of these animals." The Apulian coast is the most southerly part of the

Italian Adriatic coast. The results of this study are in contrast to the situation presented in Fig. 2 of the review article by Dhermain et al. (1994), in which it was shown that the infection had spread over almost the entire Adriatic Sea. That claim is not supported by the cited references nor by other studies of dolphin morbillivirus infection in the Mediterranean Sea. Between 1990 and 1992 the infection spread throughout the whole of the Mediterranean Sea (Aguilar and Raga, 1993; Visser et al., 1993; Cebrian, 1995). The infection spread south-east from the Ionian Sea to the Aegean Sea (Cebrian, 1995). According to Cebrian (1995) the average speed of progression was 4.6 km/day for the whole Mediterranean Sea, and 16.3 km/day for the western Mediterranean Sea. If the infection had the same tendency to spread in a north-westerly direction, i.e., in the Adriatic Sea, as it did in the Mediterranean Sea, it should have affected dolphins in all parts of the Adriatic Sea by the spring of 1992. The distance between Zakynthos Island in the Ionian Sea, where the first reported striped dolphin was stranded in July 1991 (Cebrian, 1995), and the north-west part of the Adriatic Sea is about 1,100 km. Why then did the dolphin morbillivirus infection not spread in the Adriatic Sea? Possible explanations are: (1) markedly pelagic striped dolphins, which can travel long distances and hence spread infection, do not live in the Adriatic Sea, and (2) only bottlenose dolphins live in the Adriatic Sea, their absolute number being relatively small and their density low (Notarbartolo di Sciara and Bearzi, 1992; Gomerčić et al., 1994; Notarbartolo di Sciara et al., 1994). Furthermore, schools of Adriatic bottlenose dolphins are probably isolated and their home ranges are relatively small (Bearzi et al., 1993). It can be speculated that the moribund striped dolphin found on Korčula Island died of morbillivirus infection but that, fortunately, it did not infect resident Adriatic bottlenose dolphins. Its moribund status was probably the very reason that it was found in the Adriatic Sea, in which, as we have already stressed, striped dolphins are not resident.

Finally, it can be concluded that there is no evidence that Mediterranean dolphin morbillivirus epizootic from the beginning of the 1990s, has affected the only dolphin species — the bottlenose dolphin — resident in the Adriatic Sea, although bottlenose dolphins in the Atlantic Ocean and the Gulf of Mexico have been so affected.

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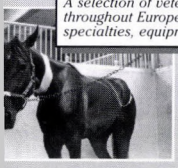

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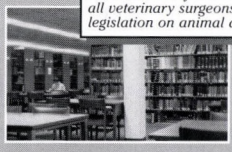

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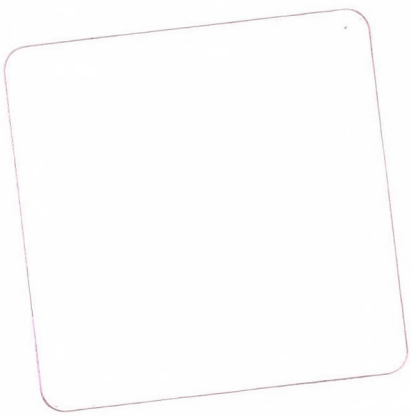

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IN VIVO ASSESSMENT OF BREAST MUSCLE, ABDOMINAL FAT AND TOTAL FAT VOLUME IN MEAT-TYPE CHICKENS BY MAGNETIC RESONANCE IMAGING

G. KÖVÉR[1], R. ROMVÁRI[1*], P. HORN[1], E. BERÉNYI[1], J. F. JENSEN[2] and P. SØRENSEN[3]

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Measurements were performed on commercial broiler chickens by magnetic resonance imaging (MRI) tomography. A total of 72 chickens were scanned at the age of 6, 7, 8, 10, 16 and 20 weeks. MRI scans based on spin echo sequence were taken in the three orthogonal planes. The volume of the pectoral muscles increased from 259 cm^3 to 1035 cm^3 in males and from 250 cm^3 to 875 cm^3 in females between 6 and 20 weeks of age. Abdominal fat volume increased from 18.9 cm^3 to 153.2 cm^3 in males and from 19.3 cm^3 to 267.0 cm^3 in females between 7 and 20 weeks of age. Total body fat volume increased from 129 cm^3 to 557 cm^3 in males and from 171 cm^3 to 1321 cm^3 in females between 6 and 20 weeks of age. The correlation coefficients between the total volume of fat pixels and the amount of abdominal fat determined in the coronal plane by MRI were 0.85 and 0.95 in the male and female sex, respectively. Changes found in the pectoral muscles (mm. pectorales) as well as in total and abdominal fat volume were in close agreement with dissection data obtained at the slaughterhouse and with laboratory data based on the measurement of total body chemical composition in both sexes. This MRI method can be a good complement to computed tomography (CT) scanning in the transversal plane.

Key words: Chicken, magnetic resonance imaging (MRI), pectoral muscle, abdominal fat, total body fat

In research concerned with the composition of animal growth, it is often necessary to measure whole body composition as well as the size, weight, and volume of organs or tissues before and during experiments comparing the effects of treatments studied. It would be ideal if these measurements could be taken on the same individuals and were non-invasive.

One of the main objectives of chicken breeders is to maximise the growth rate of lean tissue. Breast meat yield is of primary importance to the industry and

*Corresponding author

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the consumers alike. In studies with chickens (Mitchell et al., 1991), muscovy ducks (Wiederhold et al., 1994) and mulards (Wiederhold et al., 1995), it has been shown that magnetic resonance imaging (MRI) is a non-invasive technique suitable for measuring the mass of the pectoral muscle with high accuracy and permitting single or repetitive measurements during the growth period of interest.

Fat deposition poses increasing problems in broiler chicken production. Abdominal fat is of primary concern, as most of it is removed and lost during processing. Not only the quantity but also the proportion of abdominal fat in the broiler increases with growth to ten weeks of age (Leeson and Summers, 1980) or even thereafter (Sørensen and Ducro, 1994). Selection for body weight will in most cases give rise to an increase in abdominal fatness (Chambers, 1990). In addition to genetic factors, nutritional and environmental effects may also exert a significant influence on fat deposition in broilers (Leenstra, 1986).

A suitable non-invasive *in vivo* method for measuring abdominal fat, which is highly correlated with total carcass fat as demonstrated by Becker et al. (1979), would be useful in nutritional research as well as in genetic selection. A calliper technique for estimating the amount of abdominal fat in live broilers was developed and used by Pym and Thompson (1980), and phenotypic correlations between calliper measurements and the proportion of abdominal fat were 0.80 and 0.79 for males and females, respectively. Other authors obtained lower correlations by the same method or by its modifications (Moran, 1995). X-ray computed tomography (CT) was first used in poultry breeding in Norway for the prediction of fat content, with good results (for a review see Bentsen and Sehested, 1989). CT scanning can be performed only in the transversal plane, unlike MRI, which can simultaneously produce images in three parallel planes.

In the present study, MRI was used to measure the size (volume) of the pectoral muscles, and this was the first trial to measure *in vivo* abdominal and total body fat in broiler chickens ranging from 6 to 20 weeks in age and from 1420 to 5700 g in live body weight.

Materials and methods

MRI is an important method for analysing the body structure and composition of living humans and animals. This measurement procedure is non-invasive, highly sensitive, and offers the possibility to visualise the body structure in subtle detail (Stark and Bradley, 1992). MRI is based on the T_1 and T_2 relaxation processes of the excited proton (H^1) in a strong magnetic field. A radio pulse of a specific frequency is used to induce transitions between energy states by causing certain hydrogen atoms to absorb and transfer energy. Various measures of time are required for the material to return to a baseline energy state. The protons in different bounding positions have different relaxation proc-

esses and relaxation times (T_1, T_2). The relaxation times can be translated by a computer program to a visual image on the screen of the computer. The parameters of the MR image (picture) are set by a selection of a pulse sequence. The sequence applied in the present study is a T_1 weighted sequence (spin echo sequence). On T_1 weighted images substances causing high signal (i.e. bright white pixels) include fat. Other soft tissues have an intermediate level of signal (i.e. pixels with grey shading).

As the relative amount of water and the protein and lipid contents vary from tissue to tissue, so do the relaxation times (T_1, T_2). In addition, water and fat have a profound effect on the relaxation parameters (T_1, T_2). Therefore, this method has great potential in the body composition analysis of animals, similar to the revolutionary breakthrough brought about by the use of MRI in human radiology.

In the present growth experiment project commercial broiler chickens (Arbor Acres Standard) were scanned *in vivo* in a Siemens Magnetom SP 63 1.5 Tesla whole body MRI scanner. The chickens were selected from a big population at the age of 6, 7, 8, 10, 16 and 20 weeks and at a liveweight range of 1420–5700 g. From each age category, 6 male and 6 female birds representing the average weight of the groups were scanned. The details of rearing and management of the broilers have been described by Horn et al. (1998).

Before being placed into the MRI tomograph, the chickens had been starved for 10 h and anaesthetised to reduce the motion artefacts on the MR images. Anaesthesia was induced with Calypsovet (100 mg/ml concentration, containing ketamine) administered in a dose of 20 mg/kg body weight.

The chickens were moved into the tomograph in a cradle specially developed for this purpose. They were laid on their belly, with their wings close to the body and the legs tied and stretched backwards. Four series of pictures were taken. The first one was taken by the use of gradient echo sequence, which resulted in six poor-quality pictures (Fig. 1).

These transversal slices were used to localise the sagittal (Fig. 2) and coronal (Fig. 3) slices. They were followed by a series of transversal slices (Fig. 4).

All the images were taken with a body coil using a T_1 weighted spin echo sequence. The slice thickness was 8 mm in the sagittal and coronal, and 10 mm in the transversal plane, covering the whole body. Other parameters of the applied sequence were:

- number of excitations: 3,
- time of repetition: 450–820 ms, depending on the number of slices,
- time of echo: 15 ms.

The total time of acquisition depended on the number of slices necessary to cover the body. As shown in Figs 2–4, T_1 weighted spin echo sequence results in a very good picture quality with subtle details of the soft tissues.



Fig. 1. Transversal slice. Gradient echo sequence. Used for localisation. The motion artefact is caused by the breathing of the bird

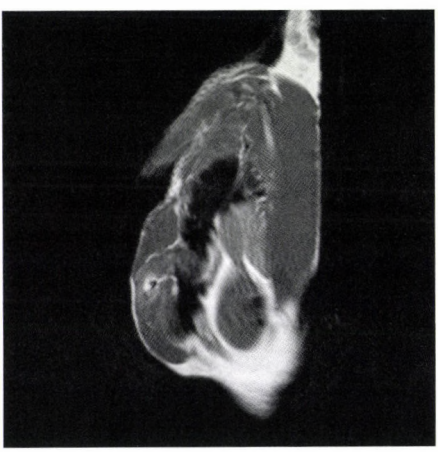

Fig. 2. Sagittal slice

The MRI picture consists of 256 × 256 pixels, each having a numeric value. High-value pixels are represented with white on MRI pictures and mean fat tissues. Pixels with lower values are represented with a grey scale and they depict muscle tissue. Unfortunately, the values of pixels from two MRI pictures cannot be compared because of the principles of MRI. Even so, some data collection is possible. With the help of the MRPC (computer program, PANNON University, Diagnostic Centre, Kaposvár) on a graphic workstation the area of fat tissues from a whole picture, or from a part of a picture can be taken. The MRI picture is very rich in details of the muscle. That is why it is possible to draw around e.g. the breast muscle.

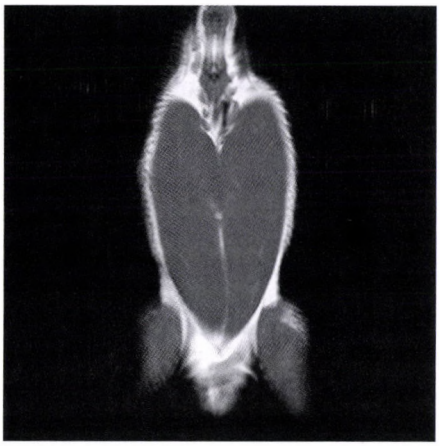

Fig. 3. Coronal slice. Used for getting breast muscle, abdominal fat deposit area and the total volume of the body fat

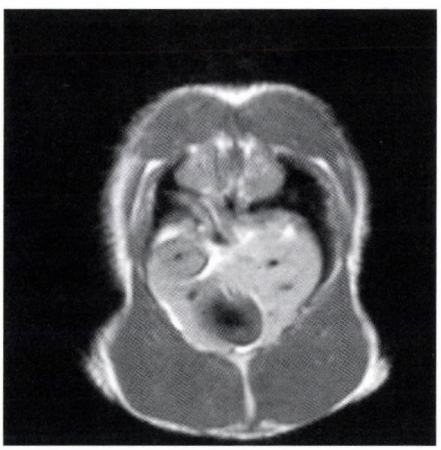

Fig. 4. Transversal slice. Spin echo sequence

The coronal plane was selected for measuring the volume of the breast muscle (m. pectoralis major et minor on both sides of the keel), since the extension of the muscle could not be determined properly on images from the other two planes. The abdominal fat depots were also clearly visible in the coronal plane, and they could not be segmented in either of the other two planes.

The following data sets were collected from the MR images of the broilers: The breast muscle area from the coronal images. The total volume of the breast muscles can be approximated by adding up the muscle areas and multiplying the sum by the slice thickness (8 mm). The amount of the abdominal fat

deposit was also determined with the help of the images obtained in the coronal plane. The two parts of the abdominal fat deposit are clearly seen in some pictures of the coronal series. Due to the motion artefacts there are some incomplete cases. In the case of the 6-week-old birds it was impossible to collect data on abdominal fat from the MRI pictures due to the insufficient amount of fat. The total volume of the body fat was also determined from scans taken in the coronal plane.

Results and discussion

On the scatterplots shown in Figs 5–6 the relationship of total fat volume and abdominal fat volume can be seen in separate charts due to the different pattern of fat development in the two sexes. Pooling data on the two sexes, the coefficient of correlation between the amount of abdominal fat determined by MRI and the total volume of fat pixels collected from all pictures covering the whole bird in the coronal plane is 0.93 ($R^2 = 0.86$). In the separate male and female groups the correlation values are 0.85 and 0.95, while the corresponding R^2 values are 0.72 and 0.90, respectively.

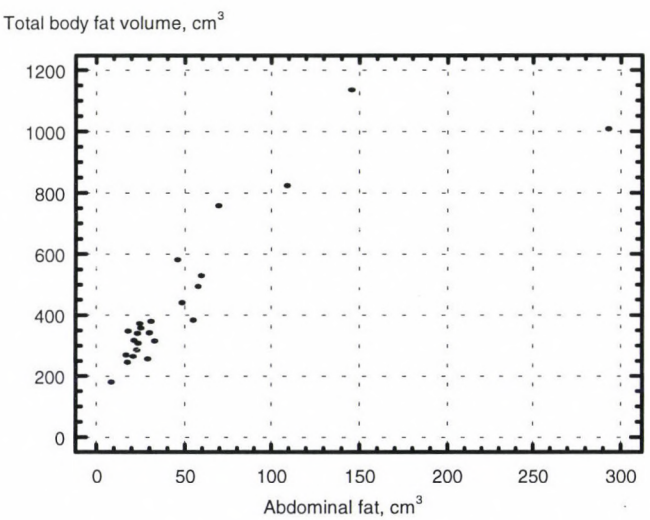

Fig. 5. The relationship between abdominal fat and total body fat volume measured by MRI. Males

The average values and standard deviations of the measured traits were calculated individually in each experimental group in both sexes (Tables 1–2).

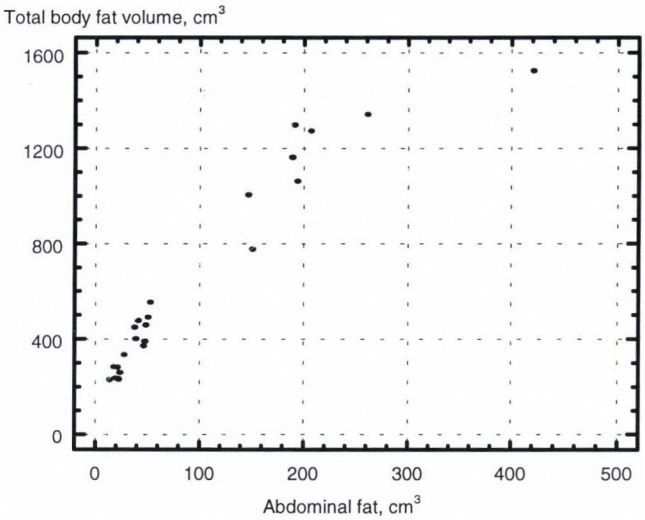

Fig. 6. The relationship between abdominal fat and total body fat volume measured by MRI. Females

Table 1

Means and standard deviations for pectoral muscle, abdominal fat and total body fat. Males

Age weeks	Volume of m. pectoralis, cm^3		Volume of abdominal fat, cm^3		Volume of total body fat, cm^3	
	Mean	SD	Mean	SD	Mean	SD
6	259	4.8			129	36.5
7	347	23.7	18.9	6.0	271	59.9
8	455	29.3	33.9	12.2	317	48.9
10	492	40.8	32.2	16.5	385	68.2
16	876	41.7	85.8	41.0	767	213.0
20*	1035	62.4	153.2	131.9	557	319.0

*Because of motion artefacts only four chickens remained in the data set

Figures 7–9 present the average values of the three measured traits. In Fig. 7 the different growth phases of pectoral muscle volume can be seen. After its intensive increase up to the age of 8 weeks, the volume of pectoral muscle continues to grow, albeit at a slower rate.

The amount of the abdominal fat deposit increases during the life stage of interest. A difference can be observed between males and females in abdominal fat volume (Fig. 8).

Table 2

Means and standard deviations for pectoral muscle, abdominal fat and total body fat. Females

Age weeks	Volume of m. pectoralis, cm^3		Volume of abdominal fat, cm^3		Volume of total body fat, cm^3	
	Mean	SD	Mean	SD	Mean	SD
6	250	21.9			171	24.6
7	331	16.2	19.3	3.7	269	35.2
8	391	37.4	36.7	11.2	346	67.9
10	449	46.7	46.2	6.5	486	41.4
16	728	95.0	191.7	41.7	1126	218.9
20	875	77.2	267.0	133.4	1321	185.6

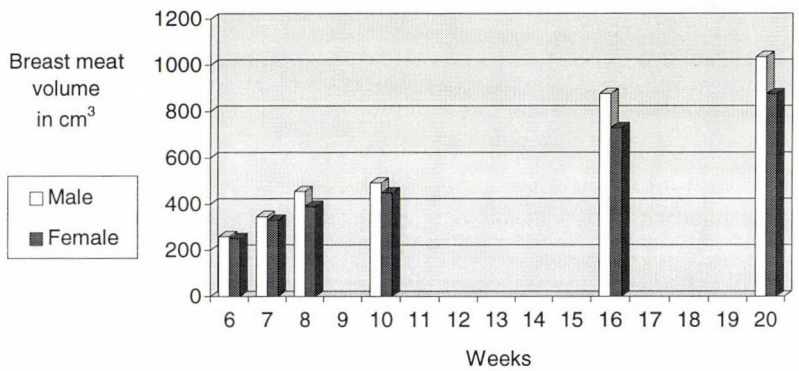

Fig. 7. Change of pectoral muscle volume measured by MRI during growth in both sexes from 6 to 20 weeks of age

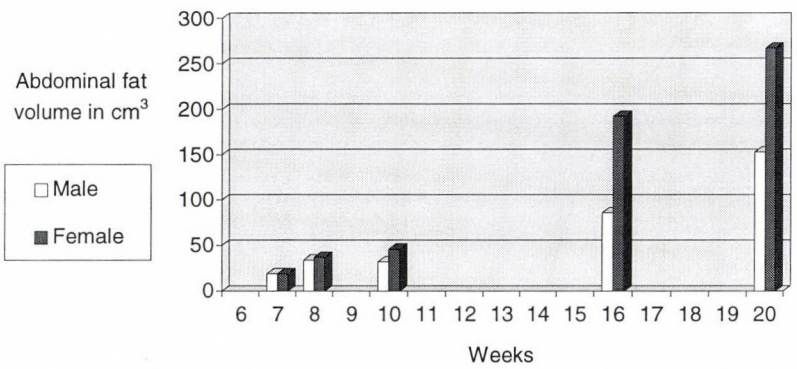

Fig. 8. Change of the volume of abdominal fat deposit measured by MRI during growth in both sexes from 6 to 20 weeks of age

The volume of abdominal fat changes parallel to the overall fat content of the body during the growth period. In males a decrease in total body fat was observed after the age of 16 weeks (Fig. 9).

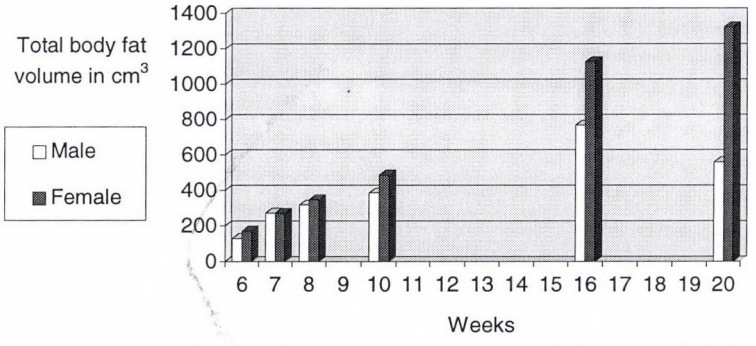

Fig. 9. Change of total body fat volume measured by MRI during growth in both sexes from 6 to 20 weeks of age

The characteristics of the three traits measured by MRI are in very close correlation with slaughterhouse and laboratory data derived from parallel experiments, using the same birds, conducted within this project (Sütő et al., 1998).

Evaluation of the MR images revealed that scans from the coronal plane proved to be the best for measuring the volume of the pectoral muscles as well as abdominal fat deposition. The applied sequence resulted in pictures of excellent quality and very high resolution.

Acknowledgements

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THYROID VOLUMETRIC MEASUREMENT AND QUANTITATIVE THYROID SCINTIGRAPHY IN DOGS

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Thyroid volumetric measurement combined with quantitative ^{99m}Tc-pertechnetate thyroid scintigraphy was performed in 62 clinical canine patients having suspected thyroid abnormalities. Euthyroid dogs (n = 22) had a total thyroid size of 3.60 ± 1.36 cm^3, the thyroid/salivary gland region of interest (ROI) ratio was 2.01 ± 0.55, the thyroid/background ROI ratio was 3.86 ± 0.90, and 20-min thyroid radioactivity uptake was 1.17 ± 0.71% of the injected dose (I. D.). By Student's unpaired test, thyroid size of the hypothyroid group (n = 36) was not statistically different from that of the euthyroid dogs, but all other quantitative data (e.g. thyroid/salivary gland ROI ratio = 1.08 ± 0.56, thyroid/background ROI ratio = 2.32 ± 0.70, and 20-min thyroid radioactivity uptake = 0.34 ± 0.22% of the I. D.) were significantly ($p < 0.001$) lower in hypothyroid than in euthyroid dogs. Evaluating the above-listed quantitative data of separated thyroid lobes by Student's paired test, there was no significant difference between the left and the right lobe either in the euthyroid or in the hypothyroid group.

Key words: ^{99m}Tc-pertechnetate quantitative thyroid scintigraphy, thyroid size evaluation, volumetric measurements, TRH stimulation test, skin biopsy, euthyroid, hypothyroid, hyperthyroid, thyroid malignancies, dog

Thyroid abnormalities are the most commonly diagnosed endocrine disorders in dogs (Paradis et al., 1991; Chastain and Panciera, 1995). The clinical symptoms of thyroid diseases are numerous, variable, of low specificity, and rarely pathognomonic; biochemical examinations (total cholesterol, TCh; creatine phosphokinase, CK; complete blood cell count, CBC) can support the clinician's suspicion and provide additional data, but the final diagnosis cannot be reached without thyroid hormone (thyroxine, T_4; triiodothyronine, T_3) measurements. Thyroid scintigraphy is also widely used in both human and veterinary medicine, because by other diagnostic imaging techniques (X-ray, ultrasonogra-

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phy) thyroid imaging is often difficult, needs expensive instrumentation and an experienced examiner but yields only morphological data.

All investigators agree with the primary need of plasma thyroid hormone measurements in canine thyroid diseases. Some endocrinologists, however, state that the biologically active hormone (T_3) levels inadequately represent thyroid gland function (Jeffers, 1990). The basis of this statement is that, unlike T_4 which is produced entirely by the thyroid gland, 40–80% of T_3 is produced from the peripheral conversion of T_4 to T_3. The remaining portion of T_3 is produced by the thyroid gland. T_3 is produced at the site of its action, i.e. predominantly intracellularly, so its activity may not be accurately reflected by serum levels. Furthermore, triiodothyronine levels in the plasma often decrease only at a late stage of hypothyroidism, and liver diseases, certain drugs etc. can sometimes give rise to false diagnosis because of their anti-T_3 effects (Jeffers, 1990).

To distinguish between primary (thyroid) and secondary (hypophyseal) hypothyroidism, the TSH response test is widely used because of its well-recognised clinical usefulness (Oliver and Held, 1985; Jeffers, 1990; Paradis et al., 1991; Hammerling et al., 1992; Chastain and Panciera, 1995; Rijnberk, 1996). Although the TRH stimulation test is known to be a less consistent tool (Paradis et al., 1991; Frank, 1996), a few investigators have reported the advantage of its use in diagnosing primary, secondary and tertiary hypothyroidism (Lothrop et al., 1984; Evinger et al., 1985; Rudas et al., 1994*a*; Grunau et al., 1995). Furthermore, TRH is less expensive than TSH. TRH is a tripeptide and its structure is the same in all mammalian species, so it may be used in companion as well as in large animals.

Histopathological evaluation of skin biopsies is an acknowledged method in the diagnostic work-up of canine hypothyroidism (Gross et al., 1992; Rudas et al., 1994*b*; Scott et al., 1995). Although it is not regarded as a definitive method for determining the thyroid status of an animal, it can serve as a surrogate test in supporting the findings of other investigations. Diseases with atrophic pattern included in the differential diagnosis of hypothyroidism are hyperadrenocorticism, castration-responsive dermatosis/growth hormone responsive dermatosis, female hyperoestrogenism, and Sertoli-cell tumour in the male (Gross et al., 1992; Rudas et al., 1994*b*; Scott et al., 1995). Few histopathological changes are suggestive of hypothyroidism: acanthosis and hyperplasia of the dermis and follicular infundibulum in the face of follicular growth cycle arrest, eosinophilic vacuolisation of arrector pili muscles, and myxoedema of the dermis, although the last two lesions are not observed in all cases (Gross et al., 1992).

Three radionuclides (^{123}I-iodine, ^{131}I-iodine and ^{99m}Technetium pertechnetate ($^{99m}TcO_4^-$) are potentially available for thyroid imaging. The choice of the ideal isotope is governed by various factors including instrumental facilities, radiation protection aspects, goals of examination and, of course, budgetary limits.

The two iodine isotopes seem to be the most specific and most suitable nuclides, as they are not only concentrated in and trapped by the thyroid but are also incorporated into thyroid hormones (organification). Unfortunately, both have major disadvantages. Because of its longer physical half-life ($T_{½}$ = 8.06 days) and β-particle emission, ^{131}I has significantly higher patient and examiner dose, and due to its hard gamma radiation (360 KeV) also needs a high-energy collimator. ^{123}I-iodine would be the best thyroid scanning agent owing to its ideal gamma energy (156 KeV with no β-particles) and physical half-life ($T_{½}$ = 13 h), but unfortunately it is too expensive for veterinary clinical purposes (cyclotron product). Currently $^{99m}TcO_4^-$ is the most widely used radiopharmaceutical in thyroid scanning because of its ideal physical properties (140 KeV gamma-emitter, no β-particles, $T_{½}$ = 6 h) and relatively low price (generator product). However, it does not organificate into thyroid hormones; it is only concentrated and trapped in the thyroid gland.

Static thyroid scanning within 1 h after $^{99m}TcO_4^-$ injection is one of the most common scintigraphic applications in veterinary practice (Hightower, 1986; Benard and Regnier, 1989; Kintzer and Peterson, 1991; Marks et al., 1994; Brawner, 1996). The dynamic aspects of thyroid scintigraphy have been less studied; still, there are a few publications on thyroid radionuclide uptake and calculated ROI ratios (thyroid/salivary gland, thyroid/background) in dogs (Sjollema et al., 1989; Nap et al., 1994; Brawner, 1996; Adams et al., 1997) and cats (Beck et al., 1985). To the best of our knowledge, no publication is yet available on quantitative thyroid scintigraphy, skin biopsies and TRH stimulation tests in dogs. Thyroid volumetric measurement using an ultrasonographic method has by now become a more or less routine clinical procedure (Wisner et al., 1994), but thyroid size evaluation in dogs by scintigraphic technique is unique even in the field of veterinary nuclear medicine: only a single paper has been published on the use of this technique in hyperthyroid cats (Forrest et al., 1996).

Materials and methods

Animals

Sixty-two canine patients with suspected thyroid disorders were presented for scintigraphic examination at the University of Veterinary Science, Budapest, Hungary. Suspicion of thyroid involvement was based on medical history, clinical signs (overweight or weight loss, muscular weakness, hair and skin abnormalities, lethargy, palpable thyroid masses) and laboratory findings (TCh, CK, CBC). The age, sex, weight and breed of the animals are shown in Table 1.

Table 1

Data of the patients

	Euthyroid (n = 22)	Hypothyroid (n = 36)	Hyperthyroid (n = 2)	Malignancies (n = 2)
Age (years)	2–14	1.5–12	6, 4.5	8, 10
Sex				
(female/spayed)	10/4	24/5	2	
(male/castrated)	7/1	6/1		1/1
Body weight (kg)	11–42	7.5–80	5.4, 17	38, 32
Breed	Mongrel (6) German shepherd (5) Doberman (4) Dalmatian (2) Boxer Puli Fox terrier Schnauzer Pinscher	Puli (6), Mongrel (6) Schnauzer (5) Doberman (3) Boxer (3) German shepherd (3) Poodle (2) Pug (2) Great Dane (2) Chow Chow (2) West Highland Komondor	Dachshund Doberman	Schnauzer Mongrel

TRH stimulation test

The diagnosis of hyperthyroidism was made on the basis of plasma basal T_4 concentration measurement as described by Bellshaw and Rijnberk (1979) and resistance to stimulation by TRH (Rudas et al., 1994*a*). Briefly: basal sample collection was carried out from the radial vein between 8:00 and 10:00 a.m. after the dogs had fasted for 12 h. Further samples were collected 4 and 8 h after intravenous administration of TRH in doses of 200 µg/dog. TRH (Sigma, Germany) used for the stimulation test was diluted in sterile physiological saline at 200 µg/mL concentration by our own laboratory. One or two mL blood samples were taken into heparinised glass syringes, and after centrifugation (1000 *g*, 5 min) the plasma samples were stored at –18 °C until the measurement. Thyroxine levels were measured using a modified human radioimmunoassay kit (Institute for Isotopes Ltd., Budapest) which had been validated for canine T_4 measurement. The criteria for euthyroid status were a basal T_4 serum level between 20 and 50 nmol/L and an at least 50% increase in plasma T_4 concentration 4 h after TRH administration.

Skin biopsy

Skin biopsies from suspected hypothyroid cases were taken with a punch biopsy device from the dorsal region of excessive alopecia, avoiding areas with signs of excessive secondary pyoderma. Samples were placed into 8% buffered formaldehyde solution, embedded in paraffin, cut at 4 μm and stained with haematoxylin and eosin. All biopsy samples were evaluated for the presence of lesions compatible with endocrine dermatoses by the same person.

Scintigraphic examinations

The dogs were sedated by intravenous injection of ketamine hydrochloride — xylazine combination (SBH Ketamine, SelBruHa Ltd., Hungary — Rompun, Bayer, Germany) in a dose of 10 mg–1 mg/kg of body weight. The animals were positioned in dorsal recumbency, the detector was placed at the centre of the cervical region, and a venous catheter was inserted into the saphenous vein. A large field of view analogue gamma camera (Picker Dyna, 9010) was used with a general parallel hole collimator. Immediately before the injection the syringe was counted (predose counts). Eighty to 150 MBq ^{99m}Tc-pertechnetate (Sorin Biomedica generator) was injected in less than 1 mL as a bolus and a dynamic study was begun simultaneously. Sixty 20-second frames were collected (128 × 128 × 64) between minutes 0 and 20. After the dynamic study static pictures were taken from dorsal and left lateral view of the neck, using 500,000 cts prerequisites, and thereafter the injection syringe and the vein catheter were counted (postdose counts).

Data were evaluated using a digital data analyser system (DIAG v.7.2, Mediso, Budapest, Hungary). Twenty-min thyroid radiopharmaceutical uptake was calculated as the percentage of injected dose (predose counts minus postdose counts) evaluating the thyroid regions of interest (ROIs) in the last frame of the dynamic study. ROIs were drawn around in the two thyroid lobes, salivary glands and background region in the neck not containing large blood vessels. Thyroid sizes were estimated using a previously calibrated modified built-in program (macro).

Statistics

Student's paired *t*-test was used for evaluating the differences in quantitative scintigraphic data of the two thyroid lobes within the euthyroid and hypothyroid groups. Student's *t*-test was used for evaluating the significance of differences between the euthyroid and hypothyroid groups. Because of the low number of cases the hyperthyroid and malignancy groups were not evaluated statistically.

Results

A total of 62 dogs with suspected thyroid abnormalities were evaluated between January 1995 and October 1996. The age distribution of euthyroid and hypothyroid animals was similar and it shows that hypothyroidism is basically a disease of middle-aged dogs. The occurrence of hypothyroidism was four times higher in female/spayed female than in male/castrated dogs (29 vs. 7). The dog breeds most commonly affected with hypothyroidism were the original Hungarian breed Puli (n = 6), the Schnauzer (n = 5), and the Doberman, Boxer and German shepherd (3 each). However, the majority of dogs (13 out of 62) were mongrels. The patients' data are shown in Table 1, and typical ventral scans of the neck region are presented in Fig. 1.

The total thyroid size of euthyroid dogs was 3.60 ± 1.36 cm^3, the thyroid/salivary gland ROI ratio was 2.01 ± 0.55, the thyroid/background ROI ratio was 3.86 ± 0.90, and the 20-min thyroid radioactivity uptake was 1.17 ± 0.71% of the injected dose (I. D.) in average. There was no significant difference between the two thyroid lobes in any of the quantitative data in the euthyroid group. The basal T_4 concentration of healthy dogs was 33.13 ± 9.64 nmol/L, which increased to 53.07 ± 16.71 nmol/L 4 h after TRH administration. The quantitative data of euthyroid dogs are shown in Table 2, while the results of the TRH stimulation test in Table 6.

Table 2

Quantitative data of euthyroid dogs (n = 22)

	Left thyroid lobe	Right thyroid lobe	Total (or average)
Size (mean ± SD, cm^3)	1.69 ± 1.14	1.92 ± 0.92	3.60 ± 1.36
Thyroid/Salivary gland ROI ratio (mean ± SD)	1.97 ± 0.51	2.04 ± 0.47	2.01 ± 0.55
Thyroid/Background ROI ratio (mean ± SD)	3.78 ± 0.92	3.94 ± 0.77	3.86 ± 0.90
20-min thyroid uptake (mean ± SD, I. D. %)	0.57 ± 0.32	0.61 ± 0.27	1.17 ± 0.71

In 9 out of the 36 hypothyroid dogs absence of TcO_4^- uptake by the thyroid glands was found (bilateral non-visualisation); thus, they were excluded from further quantitative evaluation. There was no significant difference between euthyroid and hypothyroid dogs in thyroid size, but all other quantitative data (thyroid/salivary gland, thyroid/background ROI ratios, 20-min organ uptake) were significantly lower ($p < 0.001$) in hypothyroid than in euthyroid animals. The biggest differences were demonstrated in 20-min organ uptake. Nei-

ther in this group were there significant differences between the two thyroid lobes in the quantitative data. The quantitative data of hypothyroid dogs are shown in Table 3, while the results of the TRH stimulation test in Table 6. Altogether 27 out of the 36 hypothyroid patients showed skin abnormalities. Biopsy samples were taken in 24 cases. They were evaluated as (a) showing changes compatible with endocrine disorders (n = 22), (b) cases without such changes (n = 2). In group (a), there were 4 cases with lesions suggestive of hypothyroidism, while in the other dogs of this group (n = 18) no such alterations were found. Changes regarded as suggestive of hypothyroidism were eosinophilic vacuolisation of the arrector pili muscles with concomitant acanthosis and hyperplasia of the follicular infundibulum and telogenic/catagenic arrest of the hair growth cycle.

Table 3

Quantitative data of hypothyroid dogs
(n = 36, but in 9 cases there was bilateral non-visualisation)

	Left thyroid lobe	Right thyroid lobe	Total
Size (mean ± SD, cm^3)	1.85 ± 1.02	1.90 ± 0.96	3.75 ± 1.34
Thyroid/Salivary gland ROI ratio (mean ± SD)	1.04 ± 0.44	1.12 ± 0.66	1.08 ± 0.56
Thyroid/Background ROI ratio (mean ± SD)	2.27 ± 0.62	2.38 ± 0.69	2.32 ± 0.70
20-min thyroid uptake (mean ± SD, I. D. %)	0.14 ± 0.17	0.20 ± 0.13	0.34 ± 0.22

In two hyperthyroid dog patients smaller thyroids were detected than in the euthyroid (and also in the hypothyroid) dogs. However, both animals were of small size and cachectic. Other quantitative data, e.g. the thyroid/salivary gland, thyroid/background ROI ratios, and 20-min organ radioactivity uptake were many times higher in the hyperthyroid dogs than in the euthyroid (and hypothyroid) animals. The quantitative data of hyperthyroid dogs are shown in Table 4.

Histologically, the two enormously large thyroid malignancies examined proved to be an adenoma and a malignant epithelial-mesenchymal tumour, respectively. No metastases were detected in the lungs (or in the mediastinum) or anywhere else in the body. The ROI ratios were similar to those of hyperthyroid dogs but the thyroid uptake was lower. After successful surgical removal of the mixed tumour, its volume was measured and found to be 51 cm^3 instead of 58 cm^3 as determined by scintigraphy. The other dog having adenoma died 3 months after scintigraphy. Its neoplasm was measured after necropsy and found to be 62 cm^3 instead of 66 cm^3. The quantitative data of dogs having thyroid malignancies are shown in Table 5.

Table 4

Quantitative data of hyperthyroid dogs (n = 2)

	Left thyroid lobe	Right thyroid lobe	Total
Size (mean ± SD, cm^3)	0.95 ± 0.05	0.95 ± 0.15	1.90 ± 0.20
Thyroid/Salivary gland ROI ratio (mean ± SD)	14.00 ± 2.00	16.00 ± 2.00	15.00 ± 2.00
Thyroid/Background ROI ratio (mean ± SD)	16.80 ± 2.40	18.60 ± 2.20	17.70 ± 2.40
20-min thyroid uptake (mean ± SD, I. D. %)	3.15 ± 0.97	3.18 ± 0.98	6.33 ± 0.98

Table 5

Quantitative data of dogs having thyroid malignancies (n = 2)

	Thyroid mass
Size (mean ± SD, cm^3)	62 ± 5.2
Thyroid/Salivary gland ROI ratio (mean ± SD)	18.4 ± 1.4
Thyroid/Background ROI ratio (mean ± SD)	20.2 ± 1.8
20-min thyroid uptake (mean ± SD, I. D. %)	2.11 ± 1.31

Table 6

Results of the TRH stimulation tests (n = 62)

	0 h	4 h	8 h
Euthyroid group (n = 22) T4 (nmol/L), mean ± SD	33.13 ± 9.64	53.07 ± 16.71	38.13 ± 12.74
Hypothyroid group (n = 36) T4 (nmol/L), mean ± SD	11.96 ± 5.94	14.60 ± 9.41	11.53 ± 7.42

Discussion

When determining T_4 concentrations alone, a diagnostic error manifesting itself in false positive or negative results is seen in up to 50% of the cases (Rudas et al., 1994*a*; Rudas et al., 1994*b*; Paradis et al., 1995). Therefore, some form of stimulation test should be utilised to obtain a more accurate diagnosis. In

this study, TRH stimulation negativity was chosen as the criterion of hypothyroidism; however, there is a theoretical possibility of losing tertiary hypothyroid patients (Rudas et al., 1994*a*; Rudas et al., 1994*b*). The clinical diagnosis of hyperthyroidism in dogs is also difficult in many cases, especially if thyroid enlargement or malignancy is not present. One of the major differential diagnoses would be the presence of autoantibodies to T_4 and T_3; however, in our laboratory we had no possibility to measure them. These autoantibodies produce spurious results when T_4 is measured by immunoassay, often resulting in an elevated apparent concentration of thyroid hormones in affected dogs (Peterson, 1995). This explains why basal T_4 levels and the TRH stimulation test are not suitable for use in the diagnosis of hyperthyroidism and thyroid malignancies.

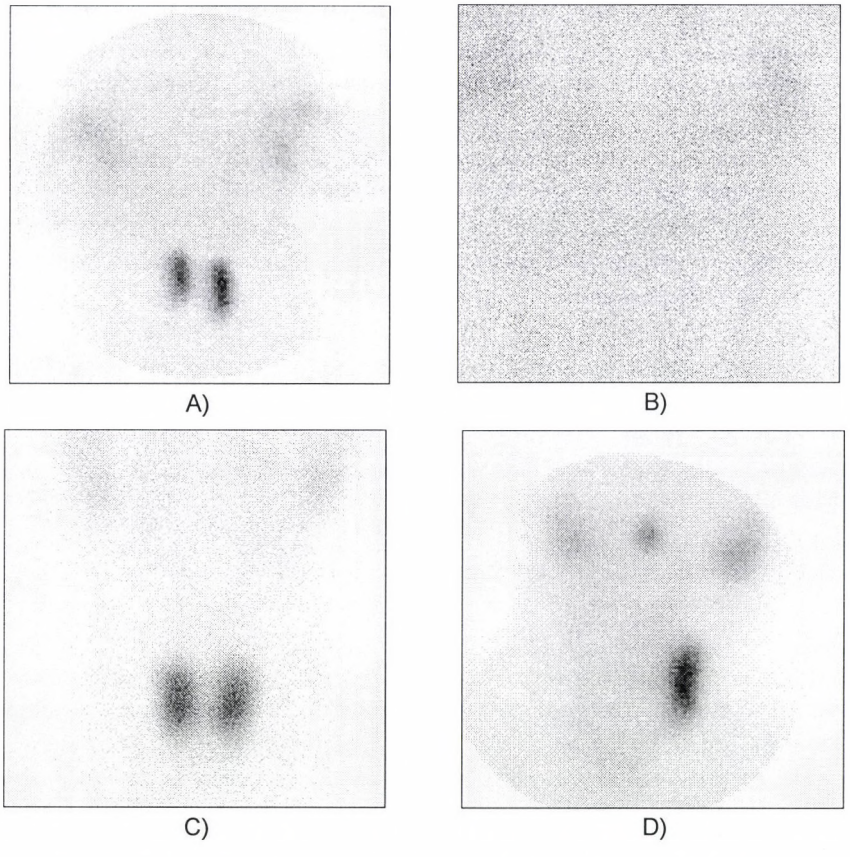

Fig. 1. Typical ventral scintigraphic images of (A) an euthyroid, (B) a hypothyroid, (C) a hyperthyroid dog, and (D) of a dog with thyroid tumour 20 min after 80–150 MBq of ^{99m}Tc-pertechnetate injection. Note differences in the radiopharmaceutical uptake of thyroid glands, salivary glands and background

The results of the histopathological examinations of skin biopsies seemed to be helpful in terms of signalling possible endocrine disorders. This method was included in this paper to underline its potential usefulness in the diagnosis of hypothyroidism.

Thyroid volumetric analysis has been elaborated by Forrest et al. (1996) for radioiodine dose calculation in cats, but no similar data are available for dogs. In nine out of 36 cases we could not estimate the thyroid size because of the absence of radiopharmaceutical uptake by the thyroid glands, and the remaining 25 hypothyroid animals did not differ significantly from the euthyroid ones. This suggests that thyroid hypofunction is independent of the volume of the thyroid glands. It was surprising also for us that, in contrast to hyperthyroid cats where thyroid volume is generally many times larger than in euthyroid cats (Wisner et al., 1994; Forrest et al., 1996; Peterson, 1995), in two of our hyperthyroid dog patients the total thyroid volume was smaller than the average thyroid volume of euthyroid and hypothyroid dogs. This might be due to the fact that our hyperthyroid dogs were extremely cachectic but both showed the characteristic clinical signs (weight loss, increased appetite, heat intolerance, restlessness, tachycardia) of hyperthyroidism.

It is a fact that static thyroid scanning is more easy to perform and does not always require the sedation of animals. The advantages of dynamic thyroid scintigraphy have been reported only in cats but not in dogs. Beck et al. (1985) estimated thyroid/salivary gland (T/S) and thyroid/background (T/B) ratios in healthy cats of different age. The T/S ratio was found to be nearly 1 and the T/B ratio was between 2 and 3. In contrast, in this study approximately twice higher T/S ratios and an average T/B ratio of 3.86 were found in euthyroid dogs. This study, however, differed from that of Beck et al. (1985) not only in the species studied but also in the time of starting the examination after $^{99m}TcO_4^-$ injection (10–15 min vs. 20 min) and in the position of background ROIs (shoulder versus neck). Although technetium pertechnetate is an isotope less frequently used for evaluating radioactivity uptake of the thyroid glands than ^{131}I-iodine, Nap et al. (1994) published the one-hour TcO_4 uptake in euthyroid (range: 0.8–3.9 %, median = 2.2%) and hyperthyroid (range 5.2–23.9 %, median = 14.8%) cats, and their data show good agreement with our results. In euthyroid dogs we obtained approximately the same quantitative data as Adams et al. (1997) in their most recent work.

In summary, we believe that simple static scintigraphy alone can help in many cases when a hypothyroid dog shows absence of thyroid radioactivity uptake or neoplastic enlargement. However, thyroid abnormalities are much more difficult to diagnose when the clinical symptoms are not sufficiently relevant and the laboratory findings are somewhere at the borderline of non-physiological values. Our results suggest that thyroid volumetric measurement and functional

$^{99m}TcO_4^-$ scintigraphy can provide valuable additional information in the diagnosis of canine thyroid abnormalities, and it is especially suitable for assessing thyroid morphology (contours, size, location, possible ectopic lobes or metastases) before surgery or chemotherapy and for evaluating thyroid function on the basis of T/S and T/B ROI ratios and the 20-min radioactivity uptake of the thyroid glands.

Acknowledgements

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DIAGNOSIS OF HYPERADRENOCORTICISM IN DOGS AS COMPARED TO HUMAN DIAGNOSTIC METHODS: A REVIEW

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Spontaneous hyperadrenocorticism is a common and well-recognised endocrine disorder occurring in dogs. The biochemical diagnosis of hyperadrenocorticism rests on the documentation of excessive glucocorticoid levels or metabolites in urine or blood. The diagnosis of canine hyperadrenocorticism is historically developed from human methods and based on non-dynamic and dynamic tests and visualisation. In some cases the different ranges of endocrine parameters or metabolites necessitate the modification of human protocols. Elevation of the activity of alkaline phosphatase (AP) and its heat-resistant isoenzyme (SIAP) induced by endogenous or exogenous glucocorticoid excess raise the suspicion of hyperadrenocorticism. Cortisol values in morning urine are related to creatinine concentrations to correct for differences in urine concentration. Theoretically, the administration of dexamethasone (DX) at a relatively low dose (0.01 mg/kg of body weight) can inhibit the pituitary secretion of ACTH and, in turn, decrease endogenous cortisol secretion for as long as 24 to 48 h. Therefore, DX administration to dogs with a functioning adrenocortical tumour would not affect the plasma cortisol concentration at any time following its administration. The high-dose dexamethasone suppression test (HDDS) is based on the observation that the function of adrenocortical tumours is independent of pituitary ACTH and they completely suppress ACTH secretion; therefore, regardless of its dose, dexamethasone is never able to suppress cortisol secretion. HDDS can be combined with the measurement of urinary cortisol/creatinine (c/c) ratio from morning urine samples on three consecutive days. In case of non-suppressible urinary c/c ratio one has to speculate on differentiating adrenal tumour (AT) from non-suppressible pituitary-dependent hyperadrenocorticism (PDH) due to a pituitary tumour arising from the intermediate lobe. Radiocholesterol scintigraphy is a less frequently used technique in the diagnosis of canine Cushing's syndrome (CCS); however, it has the same advantages in the localisation and characterisation of adrenocortical diseases as in humans.

Key words: Hyperadrenocorticism, diagnosis, humans, dogs, alkaline phosphatase (ALP), steroid-induced ALP (SIAP), urine cortisol/creatinine (c/c) ratio, adrenocorticotropic hormone (ACTH), dexamethasone suppression, scintigraphy

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Spontaneous hyperadrenocorticism is a common and well-recognised endocrine disorder occurring in dogs (Rijnberk et al., 1968*b*). The underlying cause of canine hyperadrenocorticism is most frequently adrenal hyperplasia secondary to an ACTH-secreting pituitary tumour or less frequently a primary adrenocortical tumour. The reported incidence of pituitary tumours in dogs is 85–90 per cent. Although adenomas of anterior lobe origin are most common (75%), tumours of the pars intermedia may also be associated with canine hyperadrenocorticism (Rijnberk, 1996).

The clinical features of hyperadrenocorticism are caused by abnormally high circulating concentrations of glucocorticoids. This glucocorticoid excess may also be due to exogenous administration, so hyperadrenocorticism is often iatrogenic. Suspicion of spontaneous hyperadrenocorticism arises on recognition of the clinical signs, and its diagnosis is established on the basis of abnormalities in adrenal function tests. Basically the reason for these abnormalities is either decreased sensitivity of the hypothalamic–pituitary axis to the negative feedback effects of cortisol excretion or ACTH-dependent excessive cortisol release from the adrenal cortex (Rijnberk, 1996).

The physiology of secretion and the metabolism of different adrenal steroids are similar in dogs and humans. On this basis, the dynamic adrenal cortex tests used in dogs are modified human test methods. The aim of the present review is to summarise the human diagnostic methods and their usefulness in the veterinary practice, supported by data from our own laboratory and by those from the literature.

Diagnosis of hyperadrenocorticism in human subjects

The biochemical diagnosis of hyperadrenocorticism rests on the documentation of excessive levels of glucocorticoids and/or their metabolites in urine or blood. No other tests are specific and therefore useful in diagnosis.

A. Non-dynamic tests

Urinary cortisol and cortisol metabolites. Radioimmunoassay (RIA) determination of urine free cortisol (UFC) in a 24-hour collection of urine is probably the best and simplest non-dynamic screening test for hyperadrenocorticism. The determination of unbound urine cortisol concentration is not affected by other medical conditions. The basic condition of the measurement of UFC value is the entire collected 24-hour urine output, therefore under- or overcollection of urine yields spuriously low or high results. Compliance with urinary collections causes problems, and many attempts to improve them have been made to eliminate errors of sample collection, including disturbed sleep period of patients (Crapo, 1979).

The cortisol/creatinine (c/c) ratio determined in early morning urine is a highly sensitive test. Cortisol excretion is corrected by creatinine excretion, because this value does not vary more than 15 per cent from day to day (Walker, 1979; Contreras et al., 1986). Determination of c/c ratio in 24-hour urine is not practicable because of the lack of parallelism in the excretion of cortisol and creatinine.

In addition to the measurement of UFC, there are tests largely of scientific interest to determine metabolites of cortisol such as 20-dihydrocortisol, 6-hydroxicortisol, tetrahydrocortisol and 17-hydroxicorticosteroids (17-OHCS) which reflect about 50 per cent of cortisol production (Porter and Silber, 1950; Streeten et al., 1969; Vaccia et al., 1979; Phillipon, 1982).

Plasma steroids (cortisol and adrenal androgens). Adrenal androgens such as dehydroepiandrosterone (DHA), dehydroepiandrosterone sulphate (DHAS) and androstenedione (Δ^4-A) comprise a considerable fraction of the steroid secretion of the human adrenal glands. These compounds are precursors for the synthesis of testosterone and oestrone (Haning et al., 1991*a*; Haning et al., 1991*b*). The average production of DHA and DHAS is influenced by age, namely by sexual maturation, and its secretion shows a marked age-related decrease, one-third to one-fourth in the seventh decade. DHAS has an almost exclusive adrenal origin and its plasma level is relatively stable, showing only minor diurnal variation (Rosenfeld et al., 1975). DHA is quantitatively the second most important androgen secreted by the adrenal cortex and, in contrast to that of DHAS, its level shows diurnal variation largely parallel to cortisol secretion (Rosenfeld et al., 1971).

Recent studies have revealed that the normal diurnal rhythm of plasma cortisol is influenced by environmental effects. Meal-related, stress-related increase in plasma cortisol may result in apparently elevated concentrations in normal subjects and patients with psychosis may have cortisol values one to three times higher than normal (Sacher, 1975; Follenius et al., 1982; Hamet et al., 1987; Lacroix et al., 1993). Moreover, the normal diurnal variation of plasma cortisol values in Cushing's syndrome is blunted or even absent. Elimination of the stress-related increase in plasma cortisol concentration is difficult. Sample collection from 48-hour hospitalised patients disturbs the sleep using criteria of 11:00 p.m. value or less than 8:00 a.m. value. An alternative method is multiple sampling from 1:00 P.M. till 4:00 P.M. (Desai et al., 1987).

A well-established method of testing pituitary-adrenal axis function is the determination of plasma ACTH level. Persistently undetectable levels of plasma ACTH in a patient with non-iatrogenic hyperadrenocorticism indicate a primary adrenal lesion (adenoma or carcinoma). An elevated plasma ACTH value may be caused by an ACTH-producing pituitary tumour or ectopic ACTH production. Its distinction is not reliable, therefore dynamic tests are still necessary.

B. Dynamic tests

Dexamethasone (DX) suppression tests. DX is a glucocorticoid receptor agonist, which does not cross-react with cortisol in RIA, therefore it is very useful for estimating whether the normal mechanisms of negative feedback are operative. In some human cases the reasons for abnormal cortisol production or lack of DX suppressivity are associated with the use of oral contraceptives containing oestrogen, endogenous depression, or alcoholism (Butler and Bessr, 1968; Rees et al., 1977; Nickelsen et al., 1989).

Low-dose dexamethasone suppression test (LDDS) is the best known of the dynamic screening tests for suspected hyperadrenocorticism. In the first interpretation of this test 17-OHCS excretion is measured in 24-hour collected urine during administration of 0.5 mg DX every 6 hours for 48 hours and the urine collection is repeated on the second day. 17-OHCS excretion higher than 11 μmol/day is considered to indicate hyperadrenocorticism. As there are difficulties with compliance in urine collection, the overnight LDDS is preferable (Liddle, 1960). Plasma cortisol is measured at 8:00 a.m. after an oral dose of 1 mg DX was given at 11:00 p.m. or 12:00 midnight the night before. No baseline measurement is required, and if suppression of plasma cortisol is 180 nmol/L or less, it is considered normal, while patients with hyperadrenocorticism give a different response.

The best-established dynamic test to differentiate adrenal from pituitary lesions is the high-dose dexamethasone suppression test (HDDS) (Liddle, 1960). After a baseline 24-hour urine collection, the patient takes DX, 2 mg every 6 hours, and repeats the collection of urine on the second day. Suppression of 17-OHCS to 50 per cent or less of baseline excludes adrenal adenoma and carcinoma. False-positivity occurs in up to 6 per cent of the patients with ectopic ACTH and 25 per cent of those with nodular adrenal hyperplasia (Crapo, 1979). An alternative that is suitable for differentiation is the overnight HDDS, in which plasma cortisol is measured at 8:00 a.m., the patient is given 8 mg of DX at 11:00 p.m., and another plasma cortisol determination is done at 8:00 a.m. the next day. Suppression of plasma cortisol to 50 per cent or less of baseline indicates a pituitary lesion (Bruno et al., 1985).

Metyrapone test. Metyrapone blocks 11-hydroxylation of deoxycortisol, resulting in an accumulation of 11-deoxycortisol, and plasma and urine concentrations of cortisol metabolites. Although multiple studies have confirmed that the metyrapone stimulation test well differentiates the pituitary and adrenal origin of hyperadrenocorticism, it produces side effects and the discrimination is more easily made by determination of basal plasma ACTH level (Sindler et al., 1983; Malchoff et al., 1988).

Corticotropin-releasing hormone (CRH) stimulation test. The use of ovine CRH (oCRH) stimulation for the differential diagnosis of ACTH-dependent hyperadrenocorticism relies on the assumptions (1) that corticotropinomas retain responsiveness to CRH, while noncorticotroph tumours lack CRH receptors and cannot respond to the agent, and (2) that hypercortisolism has been sufficient to inhibit the normal corticotropic response. After 1 μg/kg or 100 μg intravenous injection of oCRH the plasma is sampled for ACTH and cortisol at 15-min intervals over 2 h. Most of the patients with Cushing's disease respond with increases in plasma ACTH or cortisol, while patients with ectopic ACTH secretion do not (Malchoff et al., 1988). oCRH has been preferred to the human peptide because of greater potency, but human CRH is now more readily available and has recently been shown to offer equal sensitivity and specificity to the ovine preparation (Faria et al., 1990). Increase in mean post-CRH ACTH values (at 15 and 30 min) of at least 34 per cent indicates Cushing's disease, and an at least 20 per cent increase in the mean post-CRH cortisol value (at 30 and 45 min) indicates Cushing's disease (Nieman et al., 1993). Other studies reported an increase of at least 50 per cent in ACTH or at least 20 per cent in cortisol level (Kaye and Crapo, 1990).

Bilateral Inferior Petrosal Sinus Sampling (IPSS). The indications for the procedure are differentiating ectopic from pituitary sources of ACTH when dynamic tests are contradictory, and lateralising a pituitary adenoma when pituitary radiology is normal but dynamic tests indicate a pituitary source (Oldfield et al., 1991).

The test exploits the normal venous drainage of each half of the pituitary gland into the corresponding petrosal sinus. Each petrosal sinus is catheterised separately via a femoral approach, and blood for measurement of ACTH is obtained simultaneously from each sinus and a peripheral vein twice before and 3, 5 and 10 min after the intravenous administration of 1 μg/kg of oCRH (Miller and Doppman, 1991). ACTH concentrations are greater in the central samples in Cushing's disease and increase after CRH administration, reflecting ACTH secretion by the corticotroph adenoma. ACTH values in the central vein do not increase after CRH. A ratio of the central to peripheral ACTH values is calculated. In contrast, pre-CRH gradients have been reported similar in both Cushing's disease and ectopic ACTH secretion, the maximal post-CRH gradients in ectopic ACTH secretion were below the respective minimal gradients in Cushing's disease (Oldfield et al., 1991). An additional advantage of IPSS is its potential ability to localise the tumour to the right or left side of the pituitary gland.

C. Visualisation and localisation of the tumour

Ultrasonography, Computed Tomography (CT) and Magnetic Resonance Imaging (MRI). Although adrenal glands can be visualised by ultrasonographic techniques, scintigraphy, CT and MRI have largely replaced ultrasonography in the evaluation of functional adrenal disease.

The advent of CT scanning of the abdomen has allowed highly sensitive detection of adrenal abnormalities. However, performing a scan before the adrenal is clearly identified as the pituitary cause of the Cushing's syndrome may be misleading. The high incidence of incidentalomas of the adrenal at post mortem has been confirmed using CT (Osella et al., 1994). Very few of these have functional significance.

CT scanning has not influenced the investigation for Cushing's disease in the way that it has revolutionised other neurosurgical problems. Its low sensitivity of 47% for ACTH-producing pituitary tumours is attributable to their small size and the failure of many of these tumours to enhance with contrast material (Kaye and Crapo, 1990; Reincke et al., 1992). That aside, a positive CT scan is still very helpful to the surgeon, particularly if MRI is not locally available.

MRI scanning performs much better than CT in the investigation of Cushing's disease. The overall sensitivity is 72% without gadolinium enhancement (Newton et al., 1989). The sensitivity can be improved using gadolinium contrast agent.

Scintigraphy. Radiocholesterol scanning performed either with ^{131}I-labelled or ^{75}Se-labelled cholesterol derivatives can be a very sensitive and specific method in the visualisation of the adrenal cortex. The technique involves the advantages of so-called physiological imaging, because after intravenous administration of both radiopharmaceuticals they incorporate into the intraglandular biosynthesis of cholesterol. This occurs over a period of several days so that repeated scintigraphic examinations must be obtained from days 1 to 7 after radiopharmaceutical administration. Normal glands are usually not imaged. In Cushing's disease bilateral uptake suggests an ACTH-dependent nodular hyperplasia of both glands or, less commonly, an ACTH-independent disease (e.g. primary pigmented nodular adrenal disease, massive macronodular hyperplasia). Unilateral visualisation of the adrenal cortex indicates most frequently autonomous cortisol-producing adenomas with contralateral suppression. Adrenal carcinoma shows bilateral low uptake, because the greater size of these tumours (relatively undifferentiated, nonfunctional characteristics) does not mean a higher production of corticosteroids (Hay et al., 1996).

Diagnosis of canine hyperadrenocorticism

Although diagnosis of canine hyperadrenocorticism is historically developed from human methods, because of the different ranges of endocrine parameters or metabolites in some cases, the human protocols have to be modified.

A. Screening tests

Alkaline phosphatase (AP) activity. The group of serum AP enzymes catalyses the hydrolysis of phosphate esters, and the most common abnormality in routine laboratory findings is an increase in serum AP activity. We have found an increased AP activity in 55 per cent of dogs with hyperadrenocorticism (Fig. 1).

Heat-resistant AP activity. It is only in the dog that the endogenous or exogenous glucocorticoid excess induces a heat-resistant isoenzyme of AP (SIAP). A simple method for distinguishing between the two isoenzymes of AP uses the greater stability of SIAP at 65 °C. The test is considered quite sensitive, however, the finding of increases in SIAP is nonspecific, SIAP may be elevated in dogs during anticonvulsant therapy, in diabetes mellitus and hepatopathies (Fig. 1). In our patients with hyperadrenocorticism 66 (± SD 24.2) per cent of their AP is specifically the steroid-induced fraction (Fig. 1) (Teske et al., 1989).

Urinary corticosteroids. The cornerstone in the diagnosis of human hyperadrenocorticism in the past decades has been the 24-hour urine sample collection to provide an integrated assessment of the amount of produced urinary corticosteroids. Traditionally it has proved to be the most reliable means of confirming a diagnosis. Although the test is informative, and despite the advantages recognised as inherent with this diagnostic tool, the cumbersome nature of collecting urine for this test has made it rarely used in dogs.

Dogs with hyperadrenocorticism have a significant increase in the quantity of 17-OHCS excreted in their urine over a 24-hour period (Rijnberk et al., 1968*b*; Siegel, 1968). Additionally, administration of exogenous ACTH or DX to normal dogs was shown to result in a respective increase or decrease in 24-hour urinary 17-OHCS excretion compared to basal excretion (Wilson et al., 1967; Rijnberk et al., 1968*a*).

Measurement of UFC includes several purification steps, such as extraction and chromatography. Therefore, these determinations are not suitable for routine purposes. Cortisol determinations in unextracted urine by radioimmunoassay can easily be performed as a routine procedure. However, these determinations suffer from interference by other steroids (Murphy et al., 1981). In cases of increased plasma concentrations of cortisol a disproportionate rise in urinary cortisol may be expected, due to an increased percentage of unbound cortisol. In unextracted urine from normal human subjects the values of immunoreactive

cortisol are about six times higher than those obtained after extraction. Most of the interfering compounds have a polarity similar to that of cortisol, which makes it impossible to eliminate them by simple solvent extraction procedures. However, removal of these compounds may not be essential for diagnostic purposes, since a considerable part of them may represent metabolites of cortisol. In addition, even after chromatography it is uncertain if purification is complete, since a great number of the steroids in canine urine are unidentified. So measurements in crude urine may be helpful, but it is necessary to establish the normal ranges for each individual assay method, because, as is seen in the literature, figures for control measurements in crude urine can vary greatly depending on the method used (Murphy et al., 1981).

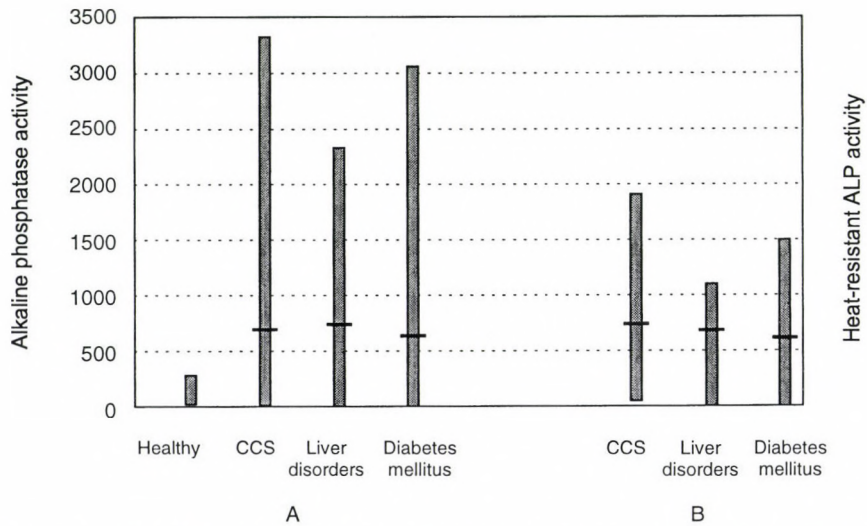

Fig. 1. Range of serum alkaline phosphatase activity (ALP) and heat-resistant ALP (SIAP) activity in healthy dogs and dogs with hyperadrenocorticism (CCS), liver diseases and diabetes mellitus. A: Mean and range of ALP in healthy dogs and dogs with hyperadrenocorticism, liver disorders and diabetes mellitus. B: Mean and range of SIAP activity in the same groups as described in graph A. Mean and range of the percentage of total serum alkaline phosphatase activity which is made up of the steroid-induced fraction were 69%, 9–100% which illustrates very well that, however, SIAP is not a specific test but a sensitive indicator of hyperadrenocorticism in dogs

By measuring cortisol in morning urine, an integration of the production over a period of about 8 h is achieved, thereby adjusting for the wide and rapid fluctuations in plasma cortisol levels. The values are related to the creatinine concentrations to correct for differences in urine concentrations. In man other

diseases can influence the cortisol and creatinine excretions. Acromegaly in man is often associated with raised urinary excretion of cortisol (Lindholm et al., 1978). Conversely, with decreased renal function, reduced clearances of cortisol and creatinine are to be expected. Dogs with hyperadrenocorticism had lower creatinine excretions than the control dogs, which can be ascribed to the loss of muscle mass in hyperadrenocorticism. It may even happen that the corticoid production as measured by c/c ratios is just around the upper limit of the reference range, but that the suspicion of the disease especially arises because of the lack of suppressibility (Fig. 2).

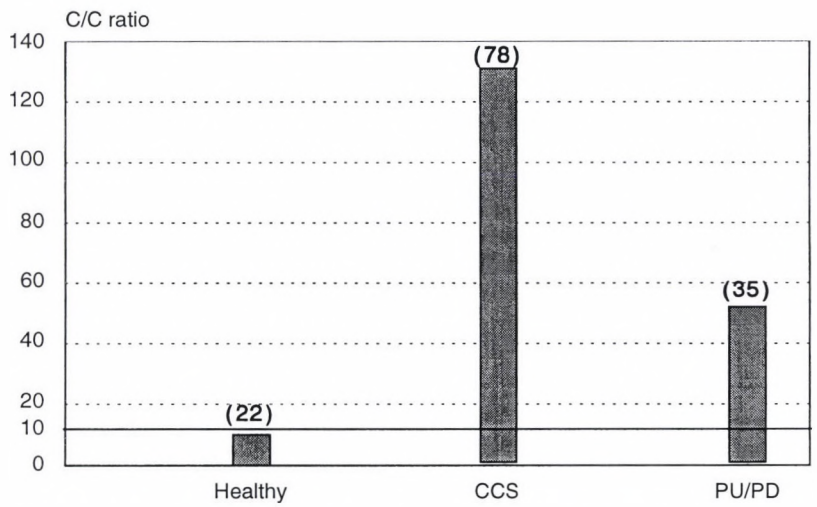

Fig. 2. Range of urine cortisol:creatinine (c/c) ratio in healthy dogs and dogs with hyperadrenocorticism and polyuria/polydipsia (PU/PD). Measurement of urine cortisol:creatinine ratio is a sensitive test for hyperadrenocorticism (CCS) but it is not specific because the clinical manifestation of polyuria/polydipsia due to other disorders disturbs the diagnosis. The number of dogs in each group is shown in parentheses

Plasma cortisol and adrenal steroids. Plasma concentrations of cortisol determined in samples obtained from normal dogs at 20-min intervals show that plasma level varies episodically, which presumably reflects episodic pituitary and adrenocortical secretion. The average number of cortisol secretory episodes in the 24-hour period was similar to the number shown to occur in humans but the frequency of cortisol peaks was equally distributed throughout the 24-hour period in dogs, in contrast to humans, who showed an increase in the number of episodes of secretion in the early morning hours, coincident with the zenith in circadian periodicity (Krieger et al., 1971). Considerable individual variability in pituitary-adrenocortical activity was apparent in the dog, like in rats, some of

which could be explained on the basis of a sex difference, in that mean levels and episodic activity of cortisol were generally greater in females. Because of the circadian rhythm and more or less sex differentiation, a 5–10 times higher basal cortisol level can be found early in the afternoon or in the evening than in samples obtained from 7:00 a.m. to 10:00 a.m., therefore basal plasma cortisol determination has little diagnostic value.

The plasma concentration of DHA in sexually mature domestic animals is an order of magnitude lower than the normal adult values in man (normal range: 200–800 μg/dl). A rise in DHA levels after sexual maturation, as is characteristic of adrenarche in man, is observed only in the rabbit and dog, and only in the dog is there an accompanying rise in Δ^4-A as in man. Since DHAS levels were undetectable or around the minimum level of detection in dog, it is not possible to rule out increased DHAS levels after sexual maturation.

In normal dogs, plasma 11-OHCS values are between 1.3–9.7 μg/100 mL. In about 50 per cent of cases the basal values in dogs with hyperadrenocorticism are in a similar range (Mulnix et al., 1976).

Plasma adrenocorticotropic hormone (ACTH) and α-melanocyte-stimulating hormone (α-MSH). ACTH is cleaved in the anterior lobe from a large precursor molecule, pre-proopiomelanocortin (pre-POMC). This prohormone is ultimately processed into additional biologically active fragments. ACTH is composed of two final sequences: α-MSH and corticotropin-like intermediate-lobe peptide (CLIP). Neither of these peptides is secreted as a separate hormone in men. Canine ACTH differs in the carboxy-terminal part of the molecule by only one amino acid from the ACTH of other species (Mol et al., 1991).

In the pars intermedia of dogs there are two cell types that also can synthesise pre-POMC. One cell type is similar to the corticotropic cells of the anterior lobe, stained with anti-ACTH, and, in the other cell type, ACTH is cleaved into α-MSH and CLIP. High basal value of α-MSH indicates intermediate lobe tumour origin, which tends to be DX resistant because of the absence of cortisol receptors from the intermediate lobe. Basal plasma concentrations of ACTH in dogs with pituitary dependent hyperadrenocorticism (PDH) were considerably higher than in those with adrenocortical tumour (AT).

The mean baseline plasma ACTH concentration in healthy dogs is 45 pg/mL (reference range: 20–100 pg/mL). Endogenous ACTH concentration less than 10 pg/mL in a dog with naturally occurring hyperadrenocorticism is strongly suggestive of a functioning AT. About 35 per cent of dogs with PDH have endogenous ACTH concentrations greater than 100 pg/mL and 55 per cent have concentrations of 45 to 100 pg/mL.

B. Dynamic tests

ACTH stimulation test. The ACTH stimulation test is most informative in the diagnosis of iatrogenic hyperadrenocorticism but it has been commonly used for the diagnosis of spontaneous hyperadrenocorticism too. The test is safe, not expensive and not time consuming.

Dogs with PDH have adrenal hyperplasia secondary to chronic ACTH excess. The hyperplastic adrenals and functioning adrenocortical tumours have a capacity to synthesise excessive amounts of cortisol. Animals with PDH or AT have the potential for an exaggerated response to ACTH.

Synthetic ACTH (Cortrosyn®, Organon, USA) 0.25 mg/dog is administered intramuscularly and samples are obtained before and at least 1 h but more exactly 1, 2 and 4 h after stimulation. Alternatively, 2.2 IU/kg of body weight ACTH-gel may be used. Normal values must be established in each laboratory, however, individual laboratories obtain reasonably similar results for plasma cortisol concentration. Results were abnormal in 62 per cent of dogs with hyperadrenocorticism, making the test useful but not absolutely reliable. Results of dogs with PDH are not distinguished from those of dogs with AT. Suppression of endogenous ACTH by adrenocortical tumours results in atrophy of all normal adrenocortical tissue. Despite their autonomous function, these neoplastic cells retain surface ACTH receptors and intracellular pathways integral to a response caused by ACTH. Most of the dogs with AT have abnormally exaggerated ACTH stimulation test results (Fig. 3).

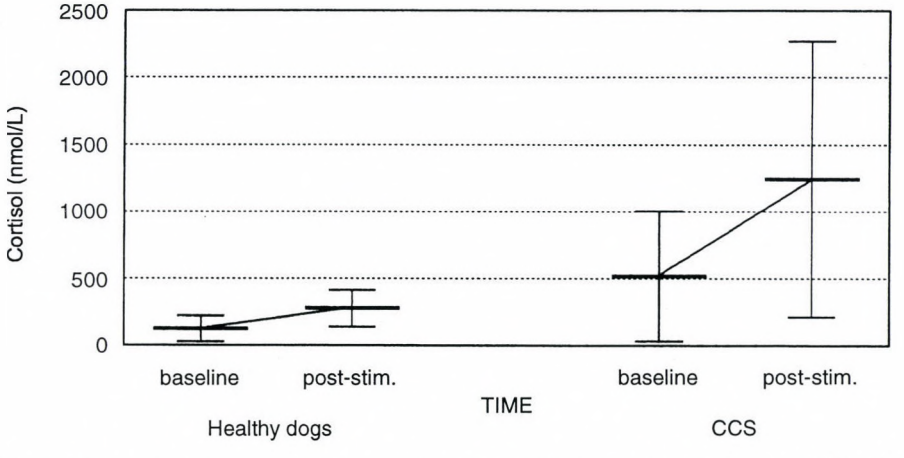

Fig. 3. Mean plasma cortisol concentrations (± 2 SD) determined before and 2 h after administration of synthetic ACTH (Cortrosyn®, Organon, USA) in healthy dogs and dogs with hyperadrenocorticism (CCS)

Dogs with clinical signs and routine laboratory test features of hyperadrenocorticism with a low baseline cortisol concentration and no response to ACTH stimulation are likely to have iatrogenic Cushing's syndrome. No other screening test differentiates naturally occurring hyperadrenocorticism from iatrogenic Cushing's syndrome.

Low-dose dexamethasone suppression (LDDS). Theoretically, the administration of relatively low dose, 0.01 mg DX/kg of body weight (Dexadresone®, Intervet, The Netherlands) can inhibit pituitary secretion of ACTH and, in turn, decrease endogenous cortisol secretion for as long as 24 to 48 h. Functioning adrenocortical tumours secrete excess cortisol autonomously, suppressing endogenous ACTH secretion. These tumours function independently of ACTH control. Therefore, dexamethasone administration to these dogs would not affect the plasma cortisol concentration at any time following administration. Chronic ACTH secretion from functioning pituitary tumours causes adrenocortical hyperplasia. The pituitary is somewhat resistant to the negative feedback effect of cortisol, and a low dose of dexamethasone causes variable degrees of suppression of plasma cortisol shortly after administration but that suppression does not last longer than 8 h (Fig. 4).

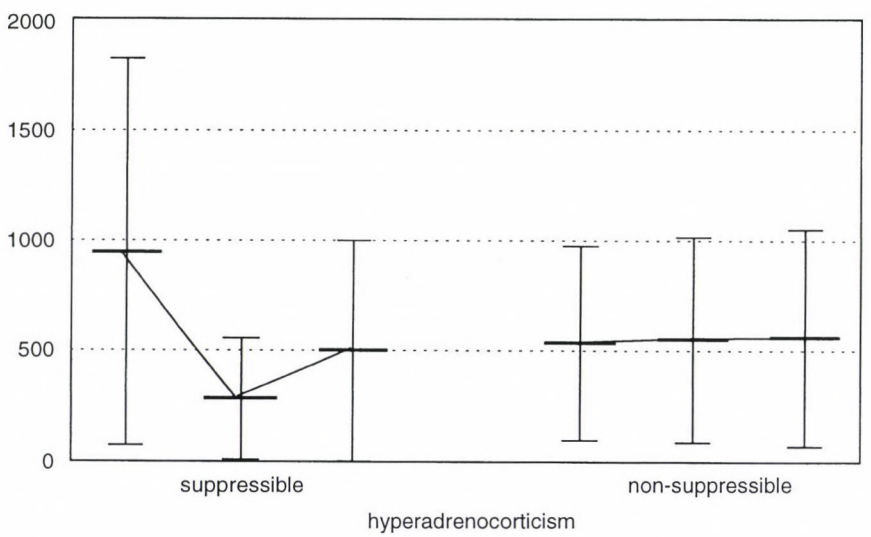

Fig. 4. Mean plasma cortisol concentrations (± 2 SD) before and following administration of low-dose (0.01 mg/kg of b. w.) dexamethasone (Dexadresone®, Intervet, The Netherlands) (LDDS) in 78 dogs with hyperadrenocorticism. These dogs were divided into two groups on the basis of suppressibility of plasma cortisol by low-dose dexamethasone. In 48 out of the 78 dogs with hyperadrenocorticism (62%), 2 h after dexamethasone administration the plasma cortisol concentration was less than 50% of the baseline concentration. Plasma cortisol level was non-suppressible to 50% of the baseline concentration in 30 dogs (38%)

Rapid dexamethasone clearance. Plasma dexamethasone concentrations in healthy dogs persist for more than 12 h. The dexamethasone clearance rates are altered in dogs with hyperadrenocorticism, which may be estimated by plasma cortisol concentrations in 8-hour samples. Healthy dogs have a plasma cortisol concentration less than 28 nmol/L 8 h after low-dose dexamethasone administration. Cortisol concentrations between 28 and 40 nmol/L have no diagnostic value and one must collect other information to determine if hyperadrenocorticism is the correct diagnosis. Seventy-nine per cent of our patients clear dexamethasone from their plasma in less than 8 h.

Differentiation of PDH from AT by LDDS. The criteria to discriminate PDH from AT are (1) a 4-hour plasma cortisol concentration less than 40 nmol/L, (2) a 4-hour plasma cortisol concentration less than 50% of basal level, and (3) an 8-hour plasma cortisol concentration less than 50% of the basal concentration. Plasma cortisol concentrations that meet one or more of these three criteria would suggest the presence of PDH.

Plasma cortisol concentrations in the fourth hour were less than 40 nmol/L in 32 per cent of dogs. Fifty-eight per cent of dogs with hyperadrenocorticism had a 4-hour plasma cortisol concentration less than 50% of baseline. Sixty-eight per cent of our patients demonstrate at least one of these criteria.

High-dose dexamethasone suppression test (HDDS). The function of adrenocortical tumours is independent of pituitary ACTH, and they completely suppress ACTH secretion; therefore, regardless of the dose, dexamethasone is never able to suppress the cortisol secretion. In contrast, chronic hypersecretion of ACTH due to PDH may be suppressed by dexamethasone in a variable and dose-dependent manner. The test protocol is similar to LDDS except that 0.1 mg DX/kg of body weight i.v. is used instead of 0.01 mg/kg. The suppression criteria are defined as a plasma cortisol concentration less than 50% of basal value or less than 40 nmol/L 4 or 8 h after administration of DX. Eighty per cent of our patients meet at least one of these four criteria for suppression on the HDDS (Fig. 5).

HDDS can be combined with measurement of urinary c/c ratio from morning urine samples on three consecutive days. Basal samples are collected on two consecutive days and the third sample is taken on the following day after administration of 0.1 mg DX/kg of body weight. The upper limit of basal urinary c/c ratio in healthy dogs is 10×10^{-6}. Exaggerated c/c ratio in basal samples suggests hyperadrenocorticism, and if the ratio in the third sample is lower than 50% of ratios in basal samples, PDH is diagnosed.

In 55 per cent of our patients the basal urinary c/c ratio (mean ± SD 39.66 ± 37.78) decreased at least 50% after administration of dexamethasone (12.25 ± 11.93), which suggests an ACTH dependency.

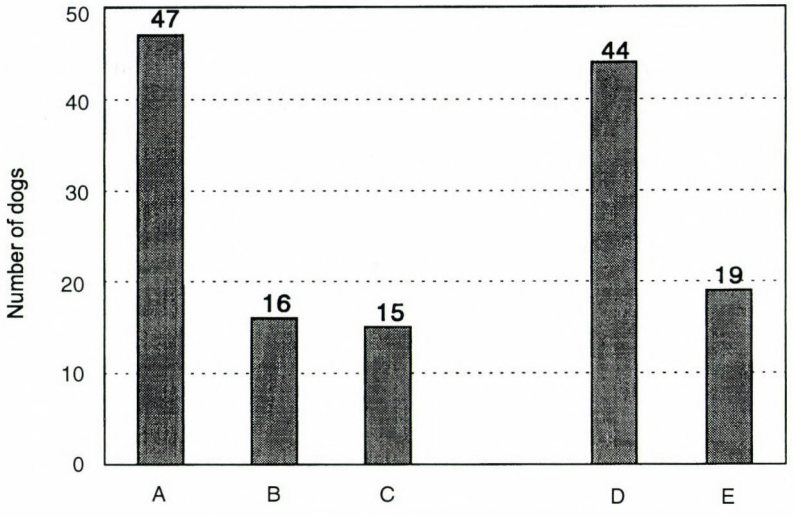

Fig. 5. Results of high-dose dexamethasone suppression test. This graph illustrates that 63 out of 78 dogs with hyperadrenocorticism (81%) showed suppression in the high-dose dexamethasone test (HDDS). Cortisol concentration was suppressed by the LDDS test in 47 dogs and there was no cortisol response to the LDDS test in 16 dogs. A: cortisol decreases in both LDDS and HDDS tests; B: cortisol level is non-suppressible by low-dose but suppressible by high-dose dexamethasone; C: no cortisol response to LDDS and HDDS; D: cortisol level is suppressed at both 4 and 8 h; E: suppression only at 4 h

In case of a non-suppressible urinary c/c ratio one has to speculate on differentiating AT from non-suppressible PDH due to a pituitary tumour arising from the intermediate lobe.

CRH stimulation and metyrapone test. A single i.v. dose of 0.1 oCRH/kg of body weight increases plasma ACTH and cortisol concentration in healthy dogs. Dogs with hyperadrenocorticism due to PDH have an elevated plasma cortisol response to oCRH; in contrast, in dogs with AT there is no significant rise in plasma cortisol level.

Oral administration of 25 mg metyrapone/kg of body weight four times in every 6 h is recommended for differentiating PDH from AT. Samples are collected before and 6 h after the final dose of metyrapone. The diagnosis of PDH can be made if metyrapone causes a decrease in the plasma cortisol concentration and a concomitant increase in plasma 11-desoxycortisol level. If both the plasma cortisol and 11-desoxycortisol concentrations decline after the administration of metyrapone, the hyperadrenocorticism is due to AT.

C. Visualisation

Computed tomography. CT is a big step in the visualisation of pituitary or adrenal glands. Unfortunately, in 50 per cent of dogs with PDH, there are no or only poorly visible microadenomas. CT scan is a non-invasive method but high expenses limit its use.

Scintigraphy. Radiocholesterol scintigraphy is a less frequently used technique in the diagnosis of canine Cushing's syndrome; however, it has the same advantages in the localisation and characterisation of adrenocortical diseases as in humans. A few days after 15–20 MBq ^{131}I-iodomethyl-19-norcholesterol administration repeated static images must be obtained. In our own experience, the normal functional adrenal cortex cannot be visualised within the first two days in dogs. Bilateral uptake was found in nodular hyperplasia (5 cases), and unilateral visualisations proved to be adenomas (3 cases), which showed the clearest imaging of adrenal glands (target/background ratio = 3, in an average) on the second day after radiocholesterol application. Based on our own data (Thuróczy et al., 1994) and on the only international reference (Mulnix et al., 1976) it can be stated that adrenocortical scintigraphy is a useful technique also in veterinary medicine.

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ENDOCRINOLOGY OF PREGNANCY: CHORIONIC SOMATOMAMMOTROPINS AND PREGNANCY-ASSOCIATED GLYCOPROTEINS: REVIEW

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The two main groups of placental proteins of ruminants are discussed in this paper: chorionic somatomammotropins (placental lactogens) and pregnancy-specific (-associated) proteins. Placental lactogens belong to the prolactin and growth hormone family. They stimulate mammogenesis, fetal growth and maternal metabolism. Pregnancy-specific proteins and pregnancy-associated glycoproteins belong to the aspartic proteinase family like pepsin, cathepsin D and E. These two groups of proteins are secreted in the maternal circulation by the binucleate cells after their migration to and fusion with the uterine cells. Their profiles were determined by radioimmunoassay (RIA). Further investigations are in progress to relate secretory profiles with alterations of the trophoblastic function such as those occurring in embryonic mortality, abortion, and fetal distress. The endocrine function of the primate and equine placenta is also discussed.

Key words: Chorionic somatomammotropins (placental lactogens), pregnancy-specific (-associated) proteins, cow, sheep, goat

The placenta and the fetus derive from an unique typical cell: the fertilised oocyte — and after cloning the reconstructed oocyte. Thereafter the fetal development is related to placental development and growth.

The structure and ultrastructure of the placenta vary considerably with species. In ruminants, the presence of binucleate cells or diplokaryocytes is characteristic. These cells derive from mononucleate cells, multiplied in fetal trophectoderm in the deep layers of the trophoblast. Binucleate cells may be recognised from day 14 after fertilisation in the ewe and from day 16–17 in the cow. They migrate towards the uterine epithelium. This migration, which continues throughout pregnancy, plays an important role in the remodelling of pla-

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cental structure and leads to a reaction with the uterine cells: the formation of a hybrid fetomaternal tissue in the ewe and goat (the synepitheliochorial) and the maternal giant cells in the cow (the trinucleate cells). Trinucleate cells are short-lived structures and syncytium is probably remodelling during pregnancy.

These multinucleate cells the cytoplasm of which contains scattered large granules, numerous ribosomes, various enzymes and a well-developed Golgi apparatus, have an important functional activity. The placenta plays an essential role in both establishment and maintenance of pregnancy as well as in fetal development, working like a respiratory, nutritional, epurative, endocrine and immunological organ. The placenta is not only a barrier, as it was thought before, but it has a real active and selective exchanger role which is developed in harmony with other systems.

The endocrine function of the mammalian placenta includes various hormones: chorionic gonadotropins, placental lactogens or chorionic somatomammotropins, prolactins, growth hormones and a series of growth factors, proteins and glycoproteins interfering with the establishment of pregnancy, corpus luteum maintenance, fetal growth, and fetomaternal recognition.

Due to a complex immunological mechanism and its regulation which have not been entirely elucidated yet, the placenta, the embryos and the fetus are protected from, or are resistant to, immunological rejection by the maternal tissues.

Since the endocrine function of the placenta has been considered to be so variable among species, a comprehensive review will be presented, based on historical references.

I. The endocrine function of the primate placenta

I. 1. Chorionic gonadotropins

In 1927, Aschheim and Zondek reported the presence of a gonadotropic factor in the urine of pregnant women and based on that discovery they developed a method for the early diagnosis of pregnancy in women (Aschheim and Zondek, 1928*a,b*). This gonadotropic factor was later called human chorionic gonadotropin (hCG).

hCG is a glycoprotein with a molecular weight of 38,400 daltons and its isoelectric point (pI) varies from 3.8 to 5.1. hCG is composed of 2 subunits: alpha and beta. The alpha subunit comprises a peptide chain of 92 amino acids, and 2 oligosaccharide chains linked to amino acids no. 52 and 78. This alpha subunit is similar to the alpha subunit of human pituitary gonadotropins. The beta subunit comprises 145 amino acids and is similar to the beta subunit of human LH; however, in hCG beta, 30 additional amino acids are located at the carboxy terminal site of the sequence. hCG beta contains several additional carbo-

hydrate chains linked to serine in positions 118–119 and 131 and to asparagine in positions 13 and 30 (Morgan et al., 1975).

The biological activity of hCG is rapidly abolished by hydrolysis with trypsin or chymotrypsin.

Due to its high content in sialic acid, the half-life of hCG is 8 hours. In early pregnancy, hCG secreted by the trophoblast before and after implantation participates in the maintenance of the corpus luteum from day 8 after fertilisation. hCG is first found in the blood from day 8–12 after fertilisation, and afterwards it appears in the urine. Its levels are high during the first trimester of pregnancy and remain detectable till parturition (Kosasa et al., 1974).

I. 2. Chorionic somatomammotropin (placental lactogen)

The human placental lactogen (hPL), also called human chorionic somatomammotropin (hCS), is a holoprotein — it is not glycosylated — composed of a single chain containing 191 amino acids and two intra-catenary disulphide bridges. Its molecular weight is 22,300 daltons but it also exists in polymeric forms with higher molecular weights.

The somatogenic activity of hPL is equivalent to only 1% of the specific activity of the pituitary growth hormone. Quantitatively, in the human placenta, hPL is one of the most important soluble proteins corresponding to approximately 10% of the total proteins. hPL is detected in the placenta 5 to 10 days after implantation (in the peripheral blood 3 to 4 weeks later). The level increases in the maternal serum as pregnancy progresses and the rate of secretion is related to the placental weight. hPL is a regulator of lipid, carbohydrate and protein metabolism. It increases the general metabolism of adipose tissue, provoking either lipolysis or lipogenesis depending on the circumstances. In pregnant women, it acts as an insulin antagonist and may be responsible for the diabetic acidosis observed during pregnancy. Like the chorionic gonadotropins, the hCS (hPL) is secreted by the syncytiotrophoblast (Watkins, 1978).

I. 3. Pregnancy proteins (Schwangerschaftsproteins)

The human placenta secretes many proteins classified as pregnancy proteins (Schwangerschaftsproteins). One of them, the pregnancy-specific B1 glycoprotein, was found to be associated with pregnancy since it is also secreted by other organs such as the gut or bone narrow. This protein is largely secreted into the maternal circulation during pregnancy and belongs to the immunoglobulin gene superfamily. These PSGs are expressed by trophoblastic cells during pregnancy. They are also over-expressed in many tumours of trophoblastic origin and fetal abnormalities (Létourneau and Beauchemin, 1997).

II. The endocrine function of the equine placenta

II. 1. Chorionic gonadotropin

In 1930, Cole and Hart showed the existence of a gonadotropic activity (pregnant mare serum gonadotropin, PMSG) in the serum of pregnant mares. Later PMSG was called equine chorionic gonadotropin (eCG). eCG (PMSG) is specific to the mare. It is synthesised by the endometrial cups which are composed of typical trophoblastic cells with one or two nuclei that migrate to the maternal endometrium between day 36 and 38 after fertilisation. These cells release the eCG during the first trimester of pregnancy and stop to do so at about day 130–140. The secretion of eCG coincides with the formation of the secondary corpora lutea in the ovaries. It represents the main luteotrophic factor responsible for progesterone ovarian secretion in the mare until the placenta becomes able to carry out this progestagen secretion.

eCG is also a glycoprotein with an apparent molecular weight between 45,000 and 64,000 daltons. It is composed of 2 subunits: alpha and beta. Its plasma half-life is about 6 days and the long duration of its action is due to its high sialic acid content (10 to 13.5%) and the length of its carbohydrate chains. Surprisingly, eCG is not secreted into urine (Cole and Hart, 1942).

hCG and eCG concentrations may be determined by various biological, immunological, immunoelectrophoretic and radioimmunological methods, by latex agglutination and haemagglutination inhibition.

II. 2. Chorionic somatomammotropins

In the horse no placental lactogen was detected (Kelly et al., 1976).

II. 3. Pregnancy-associated glycoproteins (PAG)

Recently, using molecular biology approaches, Green et al. (1994) showed the existence of pregnancy-associated glycoproteins in the equine placenta. The DNA sequence shows that equine PAG is close to the ruminant PAG and the rabbit pepsinogen.

III. The endocrine function of the ruminant placenta

III. 1. Bovine chorionic somatomammotropin (bCS) or bovine placental lactogen (bPL)

By co-culture of mouse mammary tissue and bovine cotyledonary tissue at different stages of pregnancy, Buttle and Forsyth (1976) showed that the cotyledons have a lactogenic activity. After 5 days, the amount of the lactogenic sub-

stance was about 300 ng of equivalent bovine prolactin. Several research groups (Bolander and Fellows, 1976; Beckers et al., 1980; Murthy et al., 1982) identified and purified a protein with a somatomammotropic activity in the placenta.

Bovine PL consists of multiple isoforms (M_r: 32,000 to 34,000; Murthy et al., 1982) containing N-linked and O-linked oligosaccharides (Shimomura and Bremel, 1988; Byatt et al., 1990). The placenta does not seem to secrete non-glycosylated bPL (Eakle et al., 1982) in contrast to pituitary prolactin (PRL), of which only a portion of the secreted protein is glycosylated. Using radioimmunoassays, Beckers (1983) has demonstrated that there is no cross-reaction between bPL, PRL and growth hormone (GH). Bovine PL shares 48 to 50% sequence homology with PRL but only 26 to 28% with GH (Warren et al., 1990).

Bovine PL is produced by chorionic binucleate cells (Wooding and Beckers, 1987; Milosvljenic et al., 1989; Kappes et al., 1992) which seem to deliver their product to the maternal circulation by migrating to and fusing with endometrial epithelium. Bovine PL is detectable in maternal circulation by the 4th month of gestation, but its concentration in maternal serum remains < 1 to 2 ng/ml throughout gestation. The level of this hormone in the fetus is higher (about 5 to 25 ng/ml) and shows a decline in concentration with advancing gestational age (Beckers et al., 1982; Byatt et al., 1987).

Like in sheep and goats, a bifunctional hormone secreted by the placenta can substitute for prolactin and growth hormone. Other lines of evidence indicate that mammary growth is, in part, controlled by the placenta. Both calf birth weight and placental mass are positively correlated with milk production during subsequent lactation (Erb et al., 1980; Collier et al., 1982). In cattle, specific binding sites for bPL have been identified in endometrium (Galosy et al., 1991; Kessler et al., 1991) that have little affinity for bGH or bPRL. Interestingly, endometrium, corpus luteum, and maternal and fetal liver contain binding sites (Bremel and Schuler, 1987) and mRNA encoding the bGHR and bPRLR (Scott et al., 1992). It was reported that bPL binds the bGH receptor in a 1:1 stoichiometry rather than an 1:2 stoichiometry reported for bGH, and at a different site on the receptor, raising the possibility that bPL acts through the bGH receptor in these tissues (Staten et al., 1993). Recent evidence (Scott et al., 1992) indicates that bPL competes with equal affinity with bPRL for a recombinant bPRLR. It is possible that in some adult tissues (e.g. liver and corpus luteum) bPL may exert its actions through either the bGH or bPRL receptors, through a heterodimer of bPRLR and bGHR monomers, or through a heterodimer of either one monomer of the bPRLR or bGHR and a unique receptor monomer (Anthony et al., 1995).

III.2. Ovine placental lactogen

Ovine placental lactogen (oPL) was studied and purified by Handwerger et al. (1974), Martal and Djiane (1975) and then by Reddy and Watkins (1978). However, due to the fact that the amino terminal residue of oPL was blocked, the full sequence of ovine PL had not been elucidated until quite recently (Colosi et al., 1989; Warren et al., 1990). Although the primary structure of oPL and bPL are similar in 67%, oPL has a molecular weight of 22,000 daltons and no potential N-linked glycosylation sites. Given the predominant form of oPL with a pI of 9.2, the authors suggest that oPL exists as a single isoform *in vivo.* The degree of homology between oPL and prolactin amino acid residues is higher than that of GH (49% and 28%, respectively). So, it is not surprising that oPL has both lactogenic and somatogenic activities.

Binding sites for oPL have been identified in fetal ovine liver approximately at day 60 of gestation and have been found to increase in number throughout pregnancy to reach a peak 3 to 7 days before parturition. These sites are also found in maternal liver during gestation and in the neonatal liver of one-week-old lambs. Freemark et al. (1990) demonstrated a decreased binding of oPL to fetal liver during maternal fasting, and later Freemark et al. (1992) suggested that oPL receptor might be either a variant or a shortened form of somatotropin receptor.

In trophoblastic tissue oPL is detectable at day 16 (Buttle and Forsyth, 1976). Fetal oPL concentrations decline from mid-gestation until 1 to 2 weeks before parturition. Although its fetal serum concentration and total fetal weight were not correlated (Kappes et al., 1992), the entry rate of oPL into the fetal vasculature increases with advancing gestational age (Schoknecht et al., 1992). In maternal blood, oPL can be detected at day 40 to 50 of gestation and peaks during the last trimester of pregnancy. These values are positively correlated to fetal number, milk yield and placental mass (Willard et al., 1995).

The biological activities of oPL have also been evaluated in ovarian steroidogenesis, maternal intermediary metabolism, mammary and fetal growth. Anthony et al. (1995) suggest that oPL acts through a structurally distinct receptor within the fetus, influences the fetal production of one or more insulin growth factors (IGFs), and plays an important role in fetal growth regulation.

The factors that regulate the secretion of oPL are still poorly understood but it is clear that oPL is not regulated in the same way in the fetal and maternal compartments, and nutritional factors seem to be involved. A more thorough knowledge of PL hormones will probably aid in understanding the metabolic pathways of problems occurring in late pregnancy, in postnatal development and milk production.

III. 3. Caprine placental lactogen

Caprine placental lactogen (cPL) was the first chorionic, somatogenic and prolactinic hormone described in ruminants (Buttle et al., 1972). It is secreted by the binucleate cells which are typical of the trophectoderm in ruminants during pregnancy. Several studies described the presence of prolactin-like and growth hormone-like activities in the blood of pregnant goats, and several research groups (Becka et al., 1977; Nugent and Hayden, 1987) tried to purify this protein. In the early 'nineties, cPL was purified to homogeneity (Currie et al., 1990). However, the primary amino acid sequence of cPL is not yet determined, but based on the close phylogenetic relationship of goats with sheep, it is likely that the sequence of cPL has a high percentage of similarity to that of oPL. Its molecular weight is 22,000 daltons and the pI is 8.35. Caprine PL, like oPL, seems not to be glycosylated (Byatt et al., 1992).

Caprine PL is detected from day 44 of pregnancy in jugular plasma and can be used as a late pregnancy diagnosis from day 60 (with 85% precision) (Sardajana et al., 1988).

The level of cPL increases during pregnancy. It reaches its maximum concentration in the mid- to late pregnancy, declines gradually in the 36 hours pre partum, and is undetectable by 18 h post partum (Currie et al., 1990).

PL has a significant role in the control of normal mammary development and function in goats. The increase in secretion of PL (between weeks 10 and 16) coincides with the onset of rapid lobulo-alveolar development of the mammary gland. Milk yield is correlated with the weekly mean of PL between week 11 and term (Hayden et al., 1979). Hayden et al. (1980) reported a negative correlation between the concentration of cPL and glucose while free fatty acids tended to increase with PL.

IV. Pregnancy specific proteins (PSPs) and/or pregnancy-associated glycoproteins (PAGs)

IV. 1. The bovine PSPB and PAGs

In 1982, Butler et al. isolated, from bovine extraembryonic membranes, two pregnancy-specific proteins (PSP-A and PSP-B). PSP-A was identified as α-fetoprotein, which is not strictly limited to pregnancy, and PSP-B was confirmed as specific to the placenta (Sasser et al., 1986). PSPB was initially reported to have a molecular weight of 47 to 53 kDa and a pI of 4.0 to 4.4. PSPB has never been completely characterised, although a range of molecular weights was published (Sasser et al., 1989).

In 1991, Zoli et al. purified, from bovine fetal cotyledons, a pregnancy-associated glycoprotein (PAG, later designed bPAG1). Bovine PAG1 was shown

to be an acidic glycoprotein with a 67 kDa molecular mass. Four isoforms with different isoelectric points (4.4, 4.6, 5.2 and 5.4) were detected. The pI were closely related to the amount of sialic acid of each isoform. Moreover, pI, sialic acid content and immunoreactivity were closely correlated: the most basic form being the most immunoreactive.

Molecular cloning studies showed that the PSPB amino acid sequence was homologous to that of bPAG1 (Lynch et al., 1992).

Antisera were raised in rabbits to PSPB and to bPAG1 and specific radioimmunoassays were developed (Sasser et al., 1986; Zoli et al., 1992*b*). RIAs can detect these antigens in the serum of pregnant cows, and their concentrations rise gradually throughout gestation from days 24 (Sasser et al., 1986) or 30 (Zoli et al., 1992*b*) after breeding and peak just prior to parturition, where levels as high as 542 ng/ml (Sasser et al., 1986) and 2462 ng/ml (Zoli et al., 1992*b*) have been measured. After delivery, their concentrations decreased steadily (Sasser et al., 1986; Zoli et al., 1992*b*) and the undetectable concentration was reached only by day 100 post partum (Zoli et al., 1992*b*), probably due to the long half-life of these glycoproteins, estimated to be about 9 days (Kiracofe et al., 1993). Some correlations have been shown among concentrations of bPAG1 and breed or sex of fetuses (Zoli et al., 1992*b*).

The detection of these placenta-specific antigens in the serum could be used as a specific serological method for early pregnancy diagnosis in cattle from days 28 (Zoli et al., 1992*b*; Szenci et al., 1998*a,b*) or 30 (Sasser et al., 1986; Humblot et al., 1988; Szenci et al., 1998*b*) after breeding. Detectable levels of bPAG1 have been found in about 20% of unbred heifers and non-pregnant cows and 15% of bull sera (Zoli et al., 1992*b*).

Molecular cloning studies (Xie et al., 1991) have shown that bPAG1 belongs to the aspartic proteinase family with more than 50% amino acid sequence identity to pepsin, cathepsin D, and cathepsin E. However, critical amino acid substitutions at the active site have probably made it enzymatically inactive (Xie et al., 1995).

These proteins are produced by trophoblastic binucleate cells (Xie et al., 1991; Zoli et al., 1992*a*). Another glycoprotein, structurally related to bPAG1, has been shown to be expressed in the bovine trophoblast, and it was called bPAG2 (Xie et al., 1994). Bovine PAG2, unlike bPAG1, has a catalytic centre with a consensus sequence of amino acids compatible with an enzymatic activity. Its mRNA is expressed in placenta but not in other fetal organs, and is located in some mononucleate and binucleate cells of the trophectoderm.

IV. 2. Ovine pregnancy-associated glycoproteins

The existence of antigen(s) immunologically related to bPAG1 cross-reacting with bPAG1 or bPSPB in the plasma of pregnant ewes has led to the isolation and partial characterisation of these antigens: oPAG (later designed as oPAG1) (Zoli et al., 1995) and oPSPB (Willard et al., 1995). Ovine PAG1 was initially described as having a molecular mass between 43 and 67 kDa (Zoli et al., 1995). Ovine PSPB exhibited various molecular masses from 43 to 66 kDa, with pIs ranging from 4.06 to 4.65 (Willard et al., 1995).

Molecular cloning studies have shown that oPAG1, like bPAG1, belongs to the aspartic proteinase family and shows 73% amino acid sequence identity to bPAG1 (Xie et al., 1991). Like bPAG1, it seems that oPAG1 is enzymatically inactive because of the substitution of one of the aspartic acid residues, at the catalytic domain, for glycine. Also as bPAG1, oPAG1 is synthesised by the binucleate cells of the trophoblast (Xie et al., 1991). A family of PAG-related molecules (SBU3) has been identified from ewe's placenta by Atkinson et al. (1993). These SBU3 glycoproteins with molecular masses of 69, 62 and 57 kDa showed partial amino acid sequence homology to oPAG1, bPAG1 and rabbit pepsinogen-F and cross-reacted with polyclonal antisera raised to oPAG1 or bPSPB.

Cultures of explants of ovine conceptuses or placental tissue showed that PAG or PAG-like molecules of different size were present (Xie et al., 1996) and a clear precursor-product relationship has not been established between them.

These results suggest the existence of different PAGs families in sheep placenta. These ovine antigens have been detected in the plasma of pregnant ewes by using a heterologous RIA based on bPAG1 or PSPB as tracer and standard and antisera raised in rabbits immunised against oPAG1 (Zoli et al., 1995) or oPSPB (Willard et al., 1995) as ligands.

Ovine PAG1 levels were detectable at week 3 after mating in some ewes, and by week 4 all ewes had measurable levels (Ranilla et al., 1994). Since the concentration increased slowly to week 9, subsequently there was a slow decrease to weeks 13–14. From weeks 15 to 17, the concentration increased again. Afterwards, the concentrations remained constant (around 250 ng/ml) in Merino ewes and increased in Churra ewes (from 250 to 650 ng/ml). After lambing, oPAG1 concentrations decreased rapidly and 4 weeks after parturition few ewes presented detectable levels.

oPSPB was detectable in all ewes by day 21 after breeding and its concentration increased uniformly to day 30, when values of 10.8 ng/ml were measured. Three weeks before parturition the concentration was 30.8 ng/ml and it increased to 41.6 ng/ml 1 week before parturition. Nine out of ten ewes had undetectable levels three weeks after parturition (Willard et al., 1987).

IV. 3. Caprine pregnancy-associated glycoproteins

The existence of immunologically related antigen(s) to PAG (Folch et al., 1993) or PSPB (Humblot et al., 1990) has been demonstrated in goat by using heterologous RIA, where the bovine antigens were used as tracer and standard and antisera raised to the ovine antigen.

PSPB concentrations in pregnant goats were significantly higher than those of non-pregnant goats 24 days after artificial insemination, and they rose to 40 ng/ml at the end of pregnancy. From day 25 and throughout gestation, females with 2 fetuses had higher PSPB concentrations than those with a single fetus (Humblot et al., 1990).

Recently a work has been conducted to isolate and characterise pregnancy-associated glycoprotein from caprine placenta (Garbayo et al., 1998). Briefly, the isolation procedure, monitored by a heterologous RIA, included extraction of soluble proteins at neutral pH, acidic and ammonium sulphate precipitations, gel filtration and ion exchange chromatographies. Three PAGs, which differed in amino acid sequence and apparent molecular masses (62, 59 and 55 kDa), were detected and each showed several isoforms with different pIs: $cPAG_{62}$ (pI: 5.1, 4.8), $cPAG_{59}$ (pI: 6.2, 5.9, 5.6) and $cPAG_{55}$ (pI: 5.3, 5.1, 4.9). These proteins had high sequence homology to each other and had two putative N-glycosylation sites within the 27 amino-terminal residues. This work demonstrates that PAGs are present in goat's placenta and that multiple forms are expressed.

IV. 4. Pregnancy-associated glycoproteins in other species

New members of the PAG family were found in pigs (Szafranska et al., 1995), horses (Green et al., 1994), zebras (Gan et al., 1997) and carnivores (Gan et al., 1997), showing that PAGs are not restricted to species with a synepitheliochorial placenta. In pigs, two different PAGs have been found. They share 50% sequence homology with the PAGs of ruminants.

Serologically similar antigens to PSPB and PAG1 have been found in other ruminants: mule and white-tailed deer (Wood et al., 1986; Osborn et al., 1996), mountain goats (Houston et al., 1986), red deer (Haigh et al., 1988), French Alpine goats (Humblot et al., 1990), bison (Haigh et al., 1991), moose (Haigh et al., 1993), elk (Willard et al., 1994*b*), fallow deer (Willard et al., 1994*c*) and sika deer (Willard et al., 1994*a*).

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COMPARISON OF 3H-THYMIDINE INCORPORATION AND CELLTITER 96™ AQUEOUS COLORIMETRIC ASSAYS IN CELL PROLIFERATION OF BOVINE MONONUCLEAR CELLS

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A rapid colorimetric non-radioactive assay for the determination of bovine mitogen-induced lymphocyte proliferation in cell culture was evaluated using a novel tetrazolium compound (MTS) and an electron coupling reagent (PMS) provided in the CellTiter 96™ kit (Promega). The results of the new method were compared with those of the 3H-thymidine incorporation assay using parallels obtained from the same lymphocyte population. The concentrations used in the cell suspension of primary cultured lymphocytes resulted in a significant signal/background ratio when cells were prepared from peripheral blood, spleen or mesenteric lymph nodes. The same concentrations of thymocytes resulted in a weak signal even for the highest concentrations of mitogen. A good correlation was demonstrated between the results of the two methods. The non-radioactive method performed well in assays in bovine mononuclear cells derived from prolactin-treated or -untreated calves, showing a 50% lower responsiveness to mitogenic stimulation in prolactin-deprived animals.

Key words: Bovine, lymphocyte, proliferation, non-radioactive, method

Lymphocyte proliferation is the process whereby new DNA synthesis and cell division take place in lymphocytes after a stimulus of some type, resulting in a series of changes. Mitogens such as concanavalin A (Con A), phytohaemagglutinin (PHA) and pokeweed mitogen induce proliferation in normal cells in culture. Lymphocyte proliferation can be quantified by determination of the percentage of lymphoblasts or of increased DNA synthesis. This evaluation may be achieved by the addition of a radiolabelled precursor of DNA to the culture medium and the subsequent detection of the amount of radioactivity incorporated

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into the cells during the cell cycle. The cells are separated from the suspension of culture medium by cell harvesting procedure. Addition of scintillation liquid in a next step is necessary to perform the measurement of radioactivity. The 3H-thymidine incorporation assay is a method widely used for the determination of cell proliferation (Hartzman et al., 1971; Farrant et al., 1980). Disadvantages of this method are the storage of radioactive waste of an isotope of long half-life (12.3 years) and the destruction of a non-biodegradable scintillation cocktail.

The CellTiter 96^{TM} AQueous Non-radioactive Cell Proliferation Assay (Promega) is a colorimetric method for determining the number of viable cells in proliferation or chemosensitivity assays (Cory et al., 1991; Roehm et al., 1991). The CellTiter 96^{TM} AQueous Assay is composed of solutions of a novel tetrazolium compound (MTS) and an electron coupling reagent (PMS). MTS (Owen's reagent) is bioreduced by living cells into a formazan that is soluble in tissue culture medium. The absorbance of the formazan at 490 nm can be measured directly from the 96-well assay plate without additional processing. The conversion of MTS into the aqueous soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells (Dunigan et al., 1995). The quantity of the formazan product and the amount of the 490 nm absorbance are directly proportional to the number of living cells in culture. The usefulness of the CellTiter 96^{TM} assay has been demonstrated in several systems (Cory et al., 1991; Buttke et al., 1993).

The aim of this study was to compare the 3H-thymidine incorporation assay and CellTiter 96^{TM} AQueous Assay by investigating the cell proliferation rate of bovine mononuclear cells. The performance of the non-radioactive assay was tested also in bovine mononuclear cells derived from prolactin-treated or untreated calves.

Materials and methods

Preparation of mononuclear cells from blood, spleen, mesenteric lymph node and thymus

Samples were obtained from two 5-day-old calves. Blood samples were obtained by jugular venipuncture and collected in tubes containing sodium citrate as anticoagulant. Blood was diluted 1:4 in RPMI-1640 (Sigma, St. Louis, MO) and layered 4:1 on a Histopaque solution (sp. gr. 1077, Sigma). After centrifugation at 600 g for 20 min at 4 °C, the leukocyte layer was removed from the interface. Spleen, mesenteric lymph node and thymus were minced and passed through a metal sieve screen (50 mesh, Sigma) centrifuged at 200 g. The erythrocytes contaminating the collected cells were lysed by 0.83% ammonium chloride in 20 mM Tris-HCl buffer and the remaining mononuclear cells were

washed three times in cold RPMI-1640 and finally resuspended in RPMI-1640 supplemented with 5% horse serum and 5% foetal calf serum (FCS). Cell viability was greater than 95%, evaluated by the trypan blue exclusion test. The cells were incubated at 37 °C in a 5% CO_2 atmosphere overnight. After the incubation, the cells were washed and resuspended in RPMI-1640-10% FCS.

Cell culture procedures

(a) CellTiter 96™ AQueous assay. Cells were cultured in 96-well flat bottom microtitre plates (Nunc). Seventy microlitres of RPMI-1640-10% FCS containing 1×10^5 (blood) or 2×10^5 (spleen, lymph node, thymus) mononuclear cells were added to each well. Thirty µl mitogen in RPMI-1640-10% FCS (resulting in a final concentration of Con A in the range of 0.15 to 20 µg/ml) were added to the wells. Control wells received 30 µl of RPMI-1640-10% FCS instead of mitogen. The plates were incubated at 37 °C in a 5% CO_2 atmosphere. Seventy-two hours later 20 µl/well of freshly prepared MTS/PMS solution was added to the reaction wells. The plates were then incubated for an additional 4 hours. The absorbance was recorded at 490 nm using an ELISA plate reader (Medicor, Budapest).

(b) 3H-thymidine incorporation assay. Cells were cultured in 96-well round bottom microtitre plates (Nunc). The cell culturing conditions were similar to the CellTiter assay. After 72 h of incubation, 14.5 kBq of methyl-3H-thymidine (Amersham Life Science) diluted in 20 µl RPMI-1640-10% FCS was added to each well. Following 18 h of incubation, mononuclear cells were harvested onto paper filters using a vacuum-driven multiple sample harvester system. Filters were placed in glass scintillation vials and 5 ml of scintillation cocktail (Packard) was added to each vial. Retained radioactivity was counted for 2 min/vial in a liquid scintillation counter (Beckman Instrument Scintillation Counter).

Data were expressed as mean of three replicate cultures. In order to compare the results obtained from the densitometer (i.e. OD) and the scintillation counter (i.e. CPM), the transformation of data to a relative scale was necessary. The normalisation was based on the proper non-specific binding (nsb) and the highest binding/density (B_0) on each method. The results were plotted in percentage of a relative scale.

Animal experiment

Newborn calves were divided into two groups. Calves in Group I were removed from their dams and fed Purina Nurse Chow #200™ Medicated Milk Replacer (Purina Mills, Inc., St. Louis, Missouri) with 5 mg bovine prolactin within 30 min of age and then fed Milk Replacer without added prolactin until 72 h of age. Calves in Group II were fed Purina Milk Replacer every 8 h until

72 h of age. At 72 h of age the calves were bled and euthanised with Nembutal and the spleen and mesenteric lymph nodes were removed. Mitogen assay was performed using the non-radioactive CellTiter kit after the separation of mononuclear cells.

Results and discussion

Data from the cell proliferation bioassays comparing the CellTiter 96™ AQueous assay and the 3H-thymidine incorporation method show similar results (Figs 1–3). Transformation to a relative scale is advisable to make the results comparable with other assay systems used.

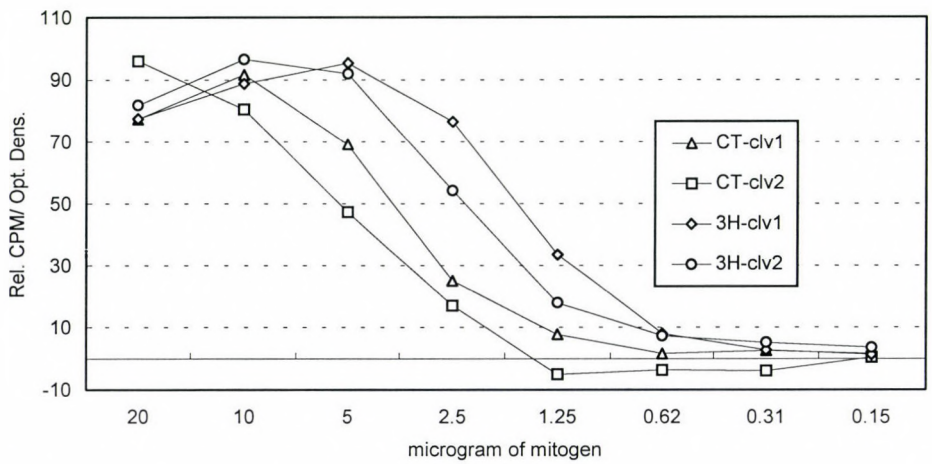


Fig. 1. Dose-response curve of concanavalin A on mononuclear cells of blood. CellTiter 96™ (CT) and 3H-thymidine (3H) assay

None of the thymic mononuclear cells derived from either of the calves were responsive in the systems used at this concentration in cell culture. When the concentration of thymocytes was increased to 200,000/well, relatively high numbers of cells interfered with the optical properties of the culture well, thus only the 3H-incorporation assay provided quality results (data not shown). Proliferation of cells in response to the mitogen Con A used in a concentration between 0.62 and 5 μg/ml shows linear parts that are parallel. According to these results, a 2.5 or 5 μg/ml Con A concentration is recommended for use in assays comparing cell proliferation of mononuclear cells resulting in a high signal/background ratio.

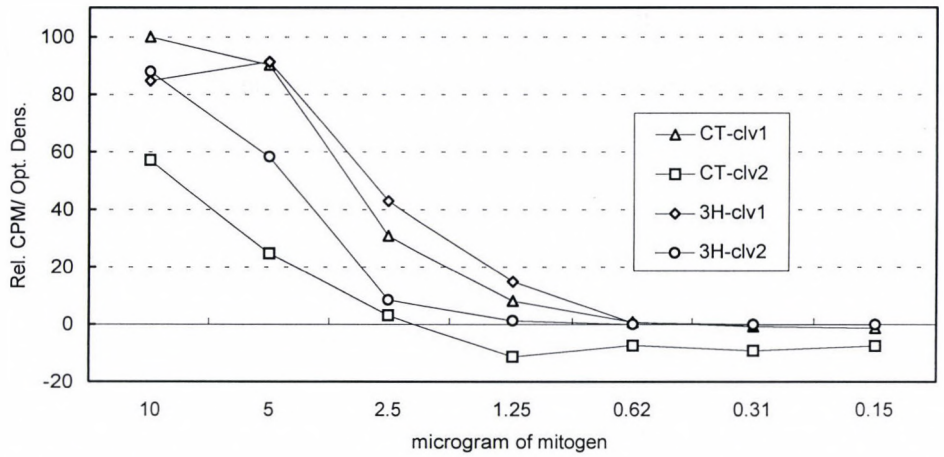


Fig. 2. Dose-response curve of concanavalin A on mononuclear cells of spleen. CellTiter 96™ (CT) and 3H-thymidine (3H) assay

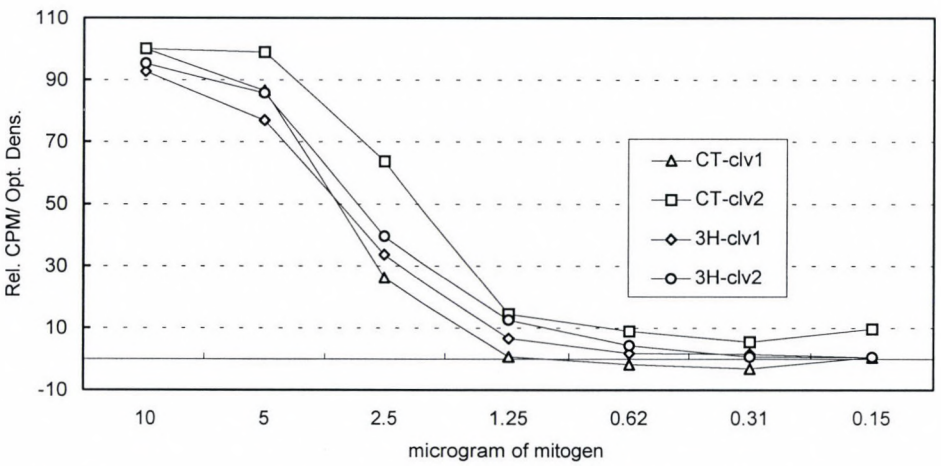
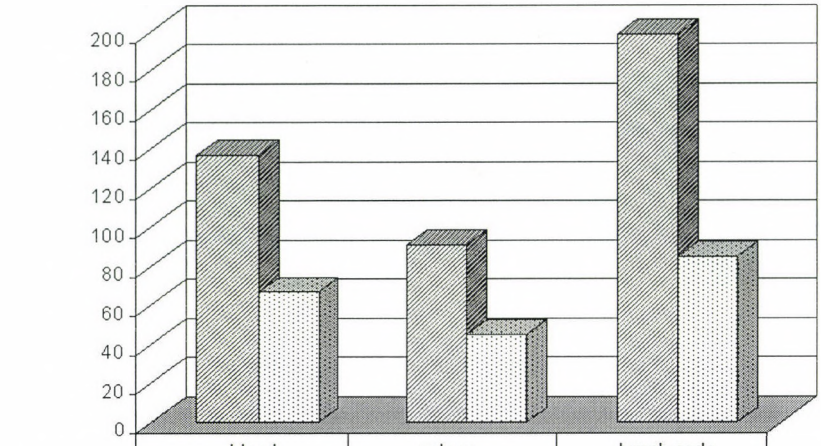


Fig. 3. Dose-response curve of concanavalin A on mononuclear cells of lymph node. CellTiter 96™ (CT) and 3H-thymidine (3H) assay

The data obtained by comparing 3H-thymidine incorporation assay and CellTiter 96™ AQueous assay indicated that the non-radioactive cell proliferation assay could be used to measure mitogenic responses of bovine mononuclear cells derived from calves. Newborn calves were used to analyse the effect of prolactin on the proliferation response to mitogenic stimulation of mononuclear cells. The results of mitogenic stimulation of mononuclear cells from calves are

presented in Fig. 4. The data show that mononuclear cells from the blood and lymphoid tissues of prolactin-deprived animals were less than 50% as responsive to mitogenic stimulation as those from calves receiving prolactin.



	blood	spleen	lymph node
Group I	137	91	199
Group II	67	45	85

Fig. 4. Concanavalin A stimulation of mononuclear cells from calves at 72 h of age

The advantages of the CellTiter 96^{TM} AQueous assay are the following: (1) non-radioactive; (2) rapid and easy performance: the assay is performed in a 96-well plate without washing or cell harvesting procedures; (3) flexible: the duration of incubation period may be adjusted to different assay circumstances resulting in different signal/background ratios. Further colour development can be achieved by prolonging the incubation period. The use of the CellTiter assay is highly recommended, and it is convenient due to the ready-to-use frozen solutions having a long shelf-life.

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VALUE OF DIFFERENT SEROLOGICAL TESTS IN THE DIAGNOSIS OF BOVINE BRUCELLOSIS IN THE AMAZONIAN REGION

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The high prevalence of brucellosis is one of the most serious animal health problems affecting cattle and buffalo herds of the Amazonian region. Modern diagnostic methods are not used or not even known, and thus test results are not available in that region. Therefore, in this study a total of 878 selected sera were subjected to comparative examination by five different serological tests (buffered plate agglutination test, tube agglutination test, complement fixation test, indirect ELISA, competitive ELISA). Indirect ELISA gave the highest number of positive results, except in samples derived from the Marajó island, for which the competitive ELISA proved to be the most sensitive. The sensitivity of the classical tests (agglutination, complement fixation) was markedly lower than that of the two ELISAs. After vaccination of 2-month-old heifer calves with the B19 vaccine, all tests showed a 50–60% seropositivity, which disappeared within four months.

Key words: Bovine brucellosis, serological tests, agglutination, complement fixation, indirect ELISA, competitive ELISA, Amazonian region

The control of bovine brucellosis is most commonly based on vaccination and/or serological testing followed by the removal of seropositive animals. Vaccination is most often done by the administration of *Brucella abortus* strain B19 to young (< 8 months old) calves. The inactivated vaccine prepared from strain 45/20 is commonly used in the final stage of eradication, and promising results have been achieved with the attenuated strain RB51 as well (Zambrano et al., 1995). Although vaccinal antibody titres disappear from most of the animals vaccinated during calfhood by the time when they reach maturity, in a relatively small proportion of animals prolonged seropositivity may occur, which is detectable for a varying amount of time by the different serological tests. This is why numerous researchers have dealt with the possibility of distinguishing the seropositivity resulting from infection from the vaccinal antibody response. The different aggluti-

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nation (AG) and complement fixation (CF) tests have proven unsuitable for that purpose. An immunodiffusion test using a cell extract designated polysaccharide B accomplished this differentiation between the two types of seropositivity for the first time (Diaz et al., 1979). However, this test was soon shown to be insufficiently sensitive and, thus, unsuitable for use as a diagnostic test (Jones et al., 1980). Seeking ways to overcome this problem, Nielsen et al. (1989) developed a 'competitive enzyme immunoassay' (C-ELISA), which was then constantly improved to meet practical demands such as sensitivity, specificity, low labour demand, and low cost (Nielsen et al., 1992; Gall and Nielsen, 1994; Nielsen et al., 1995). A similar C-ELISA technique was elaborated by Macmillan et al. (1990).

The literature contains a vast amount of data on the sensitivity and specificity of serological tests. It is a generally held view that the CF test is the most specific of the classical tests, and it also has sufficient sensitivity (Alton et al., 1975*a*). However, more and more evidence has accumulated to show that, besides its indisputably great advantages, the CF test has some properties that prevent its exclusive use as a confirmatory test. For instance, certain sera may have anti-complementary effect or show the prozoning phenomenon (McNaught et al., 1977); the test fails to detect all infected animals, i.e. is not sensitive enough (Cordes and Carter, 1979; Sutherland et al., 1982); it cannot differentiate between the vaccinal antibody response and seropositivity resulting from infection (Cunningham, 1968; Sutherland et al., 1981); and the results are subject to variation depending on the techniques used in different laboratories, i.e. standardisation is difficult (Alton et al., 1975*b*). In the light of these facts it is easy to understand why many researchers and diagnostics manufacturers started studies on the practical applicability of different enzyme-linked immunosorbent assay (ELISA) systems after the high sensitivity of that assay had been demonstrated (Engvall and Perlmann, 1971). These research efforts have elucidated numerous questions of detail, and over the past 15–20 years the ELISA has acquired a decisive role in the serological diagnosis of brucellosis as well. Naturally, the use of the classical tests will continue to be necessary in many cases, as the choice of tests is decisively influenced by the circumstances.

Opinions vary widely as to the sensitivity and specificity of the different ELISAs. Especially in the initial period of use was it concluded that the ELISA gave numerous false positive results under field conditions. Nicoletti and Tanya (1993) also stated that the ELISA was not superior to the conventional standard tests (CF test, tube agglutination). Nielsen et al. (1995) found the indirect ELISA (I-ELISA) highly sensitive but insufficiently specific while the C-ELISA sensitive and the most specific.

The purpose of this work was to compare the value of different serological tests in the diagnosis of brucellosis under conditions of the Amazonian region, and to determine the reliability of C-ELISA in differentiating between the seropositivity resulting from infection and the vaccinal antibody response.

MAGYAR
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Materials and methods

Origin of the blood samples

The blood samples used for the tests were obtained from the following eight herds.

Herd 1. The herd consisted of about 2000 cattle. The owner wanted to eradicate brucellosis, but gave up that plan after having learnt that the adult animals (n = 840) had shown 44% positivity in the CF test and I-ELISA. Based upon the results of the above two tests a total of 226 samples were selected so that all variations be represented (positive in both tests or in only one of them, negative, highly positive, inconclusive). The herd is kept under extensive conditions, and natural service is used.

Herd 2. A herd currently under brucellosis eradication and consisting of about 1000 breeding animals (total herd size: 2500 animals). In November 1995, after an acute outbreak of abortion had passed off, 42% of the cow population was seropositive by the CF test and I-ELISA. After removing the positive animals, the seropositivity was 12.4% in March 1996 and 2.4% in September 1996. Based upon the principles used in the case of herd 1, 390 out of the 3854 blood samples were selected for further testing. In addition, for animals that became positive at the second and third testing, sera collected at the first or the first and second blood samplings were also tested. This farm is also characterised by the extensive management practices typical of the whole region. The animals stay outdoors from their birth up to their death, and receive nothing more but salt supplementation in addition to grazing. However, there is an artificially planted pasture of good quality, and artificial insemination is employed. As a result, this herd is one of the best managed herds of the region.

Herd 3. The only herd from which brucellosis has already been successfully eradicated. It is a small herd comprising 193 breeding animals and their offspring (total herd size: about 500 animals). Change of seropositivity during eradication: December 1995: 22.4%, March 1996: 5.4%, June and September 1996, March 1997: no seropositive animals were found. Sera tested from this herd were as follow: 101 sera from animals giving at least three negative results by the CF test and I-ELISA, as well as sera of the 11 cows that had been negative at the first test but became positive by the second one. Twenty-one calves of the herd were vaccinated with the *B. abortus* B19 vaccine at two months old according to the manufacturer's (Brucelina B19 Vallée; Med. Vet. Gabriel Andrade Pereira Filho CRMV-MG no. 0510) recommendations. The dose of the vaccine was 5×10^{10} viable organisms, administered by subcutaneous injection. The sera of the vaccinated animals were tested by four serological tests including the buffered plate agglutination test (BPAT), CF, I-ELISA, and the tube or

serum agglutination test (SAT) at monthly intervals, parallel to those of 20 non-vaccinated control animals.

Herd 4. A herd imported from Germany in December 1994 which proved to be brucellosis free by two examinations using the CF and I-ELISA tests. From that herd, 40 samples were tested.

Herd 5. From this herd of about 1000 cows, in which irregular vaccination was practised, 54 blood samples were taken two months after vaccination with the B19 vaccine (dose: 2 ml) from heifers that had been vaccinated at 6–8 months of age.

Herds 6, 7 and 8. Samples were collected at the abattoir. No detailed data of these herds were available. All three herds were from the Marajó Island, which is the biggest alluvial island of the world, situated in the estuary of the Amazonas. It is characterised by extremely extensive animal husbandry practices: prevention, medication, and veterinary surveillance are practically non-existent; cattle and buffaloes mostly reach the slaughter weight of 400–450 kg by 6–8 years of age.

Serological tests

Buffered plate agglutination test (BPAT). This test is the most widely applied serological method in Brazil. It uses a home-produced antigen (Instituto de Tecnologia do Paraná – TECPAR, Curitiba, Paraná, Brazil). The sera are diluted in a similar manner as for the tube agglutination test. The test is read after 8 min. In this study, the BPAT was done with undiluted serum in the usual way: 30 µl of antigen was mixed with 60 µl of blood serum, the mixture was incubated at room temperature (26–28 °C) for 4–6 min, and the results were read with the unaided eye.

Tube or serum agglutination test (SAT). This was done in the usual way: starting from a 1:10 dilution, doubling dilutions were made up to 1:640. Incubation was done at 37 °C for 14–18 h, then the test was read with the unaided eye. The test was carried out by the micro-method using Takátsy's microplates. It uses a home-produced antigen (Instituto de Tecnologia do Paraná – TECPAR, Curitiba, Paraná, Brazil). A sample was considered positive if it gave agglutination in ≥ 100 IU, titres between 50 and 100 IU were regarded as inconclusive, while titres lower than that were considered negative.

Complement fixation (CF). This test was carried out as described in Public Health Monograph No. 74 of the United States Department of Health, Public Health Service (1965).

Indirect ELISA (I-ELISA). This test was performed as described by Nielsen et al. (1992). Brief description: plate: polystyrene (NUNC 69620); antigen: lipopolysaccharide; serum dilution: 1:200; conjugate: mouse Mab anti-bovine IgG_1 conjugated with horseradish peroxidase (HRPO), working dilution:

1:15,000; substrate/chromogen: H_2O_2 and ABTS (Sigma); reading at 405 nm; software: FAO/IAEA EDI program.

Competitive ELISA (C-ELISA). This test was performed as described by Nielsen et al. (1995). The reagents were kindly supplied by Dr K. Nielsen. Brief description: plate: polystyrene (NUNC 69620); antigen: hot water/hot phenol extract, lipopolysaccharide; serum dilution: 1:20 in EDTA/EGTA/Tris/Tween 20 buffer; monoclonal antibody raised in mice: M84 – specific for an epitope on the O-polysaccharide portion of lipopolysaccharide; conjugate: goat anti-mouse IgG antibody conjugated with horseradish peroxidase (commercial reagent); substrate/chromogen: H_2O_2 and ABTS (Sigma); reading at 405 nm; software: FAO/IAEA EDI program.

Results

The results of the serological tests are summarised in Table 1. Of the 878 sera, 379 were negative in all tests while 499 were positive in at least one out of the five tests used. It was surprising that only 149 out of the 499 positive samples gave a positive result in all the tests used, while the remaining 350 were negative in at least one of the tests. A relatively high number (84 samples) of inconclusive reactions (positivity in the 1:40 dilution) were obtained in the SAT. The order of decreasing sensitivity was as follows: I-ELISA (390 positive results), BPAT (317 positive results), C-ELISA (270 positive results), SAT (208 positive results), and CF (196 positive results). Markedly different results were obtained for herds no. 6–8 from the Marajó Island: 29 out of the 45 samples were positive, and only two sera gave a positive reaction in all the tests used. The C-ELISA proved to be by far the most sensitive (the C-ELISA and the I-ELISA changed places in the sensitivity ranking).

Studying the number of cases when a given sample was positive in a single test only (Table 2), a similar sensitivity ranking is obtained, with the only difference that BPAT and C-ELISA changed places: I-ELISA (93 positive sera), C-ELISA (36 positive sera), BPAT (30 positive sera), SAT (1 positive), and there was not a single case in which only the CF test would have been positive. Examining the results of the Marajó herds (no. 6–8) from this aspect, the high sensitivity of C-ELISA is even more apparent: 9 out of 12 samples were positive exclusively by this test, and only 3 sera gave a positive result in one of the other four tests (2 in BPAT and one in I-ELISA).

Since the results obtained in herd no. 1 are considered especially interesting, they are presented in Table 3 separately for the different groups of animals. It is striking that I-ELISA was by far the most sensitive when used for testing sera from pregnant heifers (group 4). Markedly different results were obtained for sera collected after calving.

Table 1

Overall results of the serological examinations using the five different tests

Serial no.	Number of sera tested	Negative in all tests	Positive							
			total	BPAT SAT CF I-ELISA	in all tests	BPAT	SAT***	CF	I-ELISA	C-ELISA
1	226	103	123	82	41	79	54 (138)	46	90	66
2	390	104	286	186	100	170	138 (39)	121	264	163
3 a*	22	8	14	12	2	6	4 (2)	7	11	7
3 b**	101	100	1	1	–	1	–	–	–	–
4	40	40	–	–	–	–	–	–	–	–
5	54	8	46	42	4	45	7 (4)	20	17	9
6	11	1	10	8	2	8	3 (4)	2	6	10
7	18	9	9	9	–	4	1 (2)	–	1	6
8	16	6	10	10	–	4	1 (1)	–	1	9
Total	878	379	499	350	149	317	208 (84)	196	390	270

BPAT = buffered plate agglutination test; C-ELISA = competitive enzyme-linked immunosorbent assay; CF = complement fixation test; I-ELISA = indirect ELISA; SAT = serum agglutination test

*3 a = those samples of animals from the brucellosis-eradicated herd that were negative by the I-ELISA at the first testing and positive at the second testing

**3 b = those samples from the above herd that were negative by the CF test and I-ELISA on two occasions

***SAT: data given in parentheses indicate the inconclusive results (agglutination titre: 1:40)

Table 2

Number of cases when the sera were positive in a single test only

Serial no.	BPAT	SAT	CF	I-ELISA	C-ELISA
1	1	–	–	21	12
2	7	1	–	68	9
3 a	–	–	–	3	2
3 b	1	–	–	–	–
4	–	–	–	–	–
5	19	–	–	–	1
6	–	–	–	–	2
7	1	–	–	1	4
8	1	–	–	–	6
Total	30	1	–	93	36

The test results obtained for the 21 calves vaccinated with the B19 vaccine at two months old are presented in Table 4. The 20 bull-calves used as control were negative on all occasions in all of the tests used.

Discussion

It is now widely known that the results of individual serological tests may show marked variation. This can be attributed to the different sensitivity of the tests as well as to the mechanism of immunoglobulin production and persistence. Despite this knowledge, the differences observed when comparing the results of individual serological tests in this study were surprising. Minor errors (such as the technician's error, imperfect fitting of much-used pipettes and pipette tips, etc.) are likely to have occurred during the mass tests performed; however, it can be ruled out with certainty that these sources of error could have exerted an influence on the overall performance of the serological tests compared in this study.

With the exception of the blood samples derived from the Marajó herds, the highest number of positive results were obtained in the I-ELISA, which was a typical finding also during the testing of an additional about 5,000 blood samples (Molnár et al., 1998). The data presented in Table 1 indicate the same trend for herds no. 2 and 3. The number of positive animals was 61.7% and 36.4% higher by the I-ELISA than by the C-ELISA in herds 2 and 3, respectively; still, there were some sera for which only the latter test gave positive results. What is even more interesting: there were two cases in both herds when the I-ELISA was negative at the first testing but became positive by the time of the second test, while by the C-ELISA these samples were positive already at the first blood sampling.

Table 3

Test results obtained for herd no. 1, broken down for different groups of animals

Serial no.	Number of sera tested	Negative in all tests	Positive							
			total	BPAT SAT CF I-ELISA	in all tests	BPAT	SAT*	CF	I-ELISA	C-ELISA
1	21	13	8	7	1	3	1 (1)	2	5	5
2	20	7	13	11	2	7	5 (7)	3	8	3
3	36	-	36	9	27	36	31 (2)	28	32	30
4	75	45	30	28	2	10	4 (7)	3	24	11
5	74	38	36	27	9	23	13 (15)	10	21	17
Total	226	103	123	82	41	79	54	46	90	66

1: Samples from dairy herd, kept separated from the remainder of the herd
2: Samples from breeding bulls
3: Samples from cows, based upon the positivity of the BPAT carried out on the spot
4: Samples from pregnant heifers, without any selection
5: Samples taken from primiparous cows 1–2 months after calving, without any selection
*SAT = data given in parentheses indicate the inconclusive results (agglutination titre: 1:40)

Table 4

Postvaccinal seropositivity of the 21 heifer calves immunised with the B19 vaccine

Time after vaccination	I-ELISA	C-ELISA	SAT	BPAT
1 month	10	13*	13*	13*
2 months	5	4	4	8
3 months	–	2	2	3
4 months	–	–	–	1

*The same 13 calves were positive in all tests, 10 of them also in the I-ELISA. At the second and third testing there no longer was a 100% agreement between the results of the tests

In our view, it is difficult to speak about the specificity of individual serological tests without a detailed and thorough bacteriological examination. By comprehensive studies using sera selected from a large serum bank, Nielsen et al. (1995) have demonstrated that the C-ELISA developed by them is highly sensitive (100% positivity was obtained for the serum of animals from which the pathogen was isolated). At the same time, this test was highly specific, too, as only a negligibly low proportion of sera obtained from negative herds gave a positive reaction. Even the latter positive reactions were mostly due to the fact that the animals had recently been vaccinated. In this study, the C-ELISA developed by Nielsen et al. (1995) was used. According to Nielsen et al. (1995), I-ELISA was highly sensitive, detecting infection in 100% of the cases, but showed a high rate of nonspecific positive reactions. In this study, the sensitivity of the I-ELISA was especially surprising as compared to that of the CF test and the SAT: the number of positive sera was nearly twice as high in the I-ELISA as in the other two classical tests. Of the data presented in Table 3, those shown in rows 4–5 are especially interesting. When used for testing sera from pregnant heifers, the I-ELISA was much more sensitive than any other test. These differences were markedly smaller or non-existent in the case of samples collected 1–2 months after calving (the highest number of positive reactions were obtained by the BPAT).

Unfortunately, in the region under study the lack of reliability is one of the biggest problems. According to the owner and the veterinarian, no vaccination was performed in herds 1–2. Judging from what the animal tenders told us, this is not at all certain (it is true, however, that any injection treatment may be mistaken for 'vaccination' and, thus, it is almost impossible to determine the truth).

The results obtained for samples originating from the Marajó Island are perhaps the most surprising. namely, in these cases the C-ELISA was consistently the most sensitive, much more sensitive than any other procedure: its se-

ropositivity rate was more than three times, five times and 12.5 times higher than that of the I-ELISA, the BPAT, and the CF test, respectively. While it is true that the low number of sera tested prevents us from drawing far-reaching conclusions, the observed phenomenon is remarkable and warrants further study. (It should be mentioned here that while no I-ELISA is available for the testing of buffalo blood samples, the testing of serum samples from Marajó buffaloes by C-ELISA and by the CF test revealed similar trends). It can be taken for certain that no vaccination has been performed in these herds, the animal husbandry practices have not changed in the past 100–150 years, and unimpeded mixing of the herds is a general feature. In view of these facts, it is possible that a chronic infection has developed which can be reliably detected by the C-ELISA alone.

The results of this study indicate that vaccination with the *B. abortus* B19 vaccine leads to an about 50–60% seropositivity rate in all tests. Subsequently the seropositivity rate decreased in all tests, and positivity disappeared after four months. Herd no. 5 (Table 1) was severely infected, and it cannot be ruled out that the 9 animals positive in the C-ELISA were infected also by a virulent strain. The number of calves vaccinated at two months old in the eradicated herd (no. 3) was also low, and further investigations are needed to determine whether the C-ELISA can distinguish the vaccinal antibody response from antibodies resulting from infection.

Mention should be made of the interesting observation that, based upon the examination of about 5,000 blood samples by different tests (Molnár et al., 1998) the I-ELISA available to us seems to be too sensitive (i.e. the cut-off point of positivity should have been set at a lower value) and, thus, it is likely to have given a positive result for some non-infected animals as well. Despite this fact, we think that this test could be a very important tool in brucellosis eradication precisely on account of its high sensitivity. This is demonstrated by the following two data: in herd no. 3 where the initial seropositivity rate had been 22.4%, all infected animals could be eliminated by two serological examinations, while in herd no. 2, where the eradication program was started a few months after an acute outbreak of abortion had passed off, the initial seropositivity rate of 42% was reduced to less than 3% by two tests. Based upon the studies carried out so far, it appears that by the use of the two ELISA procedures, combined with a suitable approach, the eradication of brucellosis could be accomplished rapidly in any region of the world.

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COMPARISON OF THE PROTEIN COMPOSITION OF *ERYSIPELOTHRIX RHUSIOPATHIAE* STRAINS OF SUBTYPE 1A ISOLATED FROM DUCKS AND PIGS

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Erysipelothrix rhusiopathiae strains isolated from duck carcasses and pigs were examined by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and the results were evaluated by a computerised method in order to compare the strains for protein composition according to molecular mass. To characterise the similarity of the strains, Pearson's correlation coefficient was calculated. The degree of similarity between duck strains originating from the same flock was 980–991, while that of strains isolated from ducks and pigs varied between 836 and 991 on a scale of 0 to 1000. The method gives highly reproducible results and can be utilised in epidemiological investigations. Its use may provide new data on *E. rhusiopathiae* strains, complementing the results of methods recommended for this purpose earlier and increasing the efficiency of epidemiological investigations.

Key words: *Erysipelothrix rhusiopathiae*, strains, duck, pig, origin, SDS-PAGE, protein analysis, Pearson's correlation coefficient

The serotyping of *E. rhusiopathiae* enabled researchers to judge the aetiological significance of strains of different type, isolated from different disease forms. The majority of strains isolated from cases of septicaemia in pigs was found to belong to subtype 1a (Kucsera, 1979; Xu et al., 1984; Eamens et al., 1988), while the chronic forms of disease yielded strains of subtype 2a (Kucsera, 1979).

Occasionally, *E. rhusiopathiae* causes mass or substantial morbidity and mortality in ducks. According to data of the literature, the serotype distribution of strains isolated from duck carcasses is as follows: subtype 1a (Sztojkov, 1971; Bisgaard et al., 1980), subtype 1b (Bisgaard et al., 1980; Eamens et al., 1988), types 2, 8 and 9 (Bisgaard et al., 1980). *E. rhusiopathiae* strains of subtype 1a have also been isolated from the pharynx, heart and intestine of healthy ducks (Xu et al., 1984; Vaissaire et al., 1995).

As *E. rhusiopathiae* strains of the same serotype could be isolated from different species of animals, it was assumed that diseased animals and those carrying the bacterium may infect animals belonging to other species. Pigs and pig

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manure were suggested as a possible source of infection in connection with diseases caused by *E. rhusiopathiae* in other species of animals (Hall, 1963; Sztojkov, 1971). According to other authors, poultry carrying *E. rhusiopathiae* may infect pigs or poultry (Xu et al., 1984). Determination of the serotype of the pathogenic strains may contribute to elucidating the source of infection (Kucsera, 1979). Other authors have found that serotyping itself is not sufficient for that purpose. In their opinion, serotyping could be useful in conjunction with multilocus enzyme electrophoresis of the strains (Chooromoney et al., 1994).

In this study, *E. rhusiopathiae* subtype 1a strains causing mortality in ducks (Sztojkov, 1971) and isolated from cases of septicaemia in pigs were examined and compared for protein composition. The objective was to determine the degree of similarity of strains originating from the above two animal species, and the potential usefulness of the results in epidemiological investigations.

Materials and methods

Bacterial strains

The *E. rhusiopathiae* subtype 1a strains examined are maintained in lyophilised state.

Strains isolated from ducks: Kacsa (Duck) 509/1; Kacsa (Duck) 344; Kacsa (Duck) 397. The strains had been isolated from three duck carcasses during an outbreak of *E. rhusiopathiae* infection in a duck flock (Sztojkov, 1971).

Strains isolated from pigs: Mezőcsát; TU; Bp. XIV; Üllő; Dánszentmiklós; Gyúró; Gyál; A 360 (see ATCC 35426).

Media and culture

The bacterial strains were cultured in a semisynthetic broth described in a previous paper (Bernáth et al., 1997), without radioisotope addition. The strains were inoculated into the broth from 24-h agar cultures, and the broth was then incubated at 37 °C for 24 h. Subsequently the bacteriological purity of the cultures was checked by inoculation onto a solid medium.

Processing of test material for electrophoresis

The bacterial cultures were centrifuged and the sediment was washed and resuspended as described previously (Bernáth et al., 1997). A bacterium suspension of approx. 500 µl volume was cooled in ice and sonicated with 50 W power for 30 sec, using a Laborsonic L (B. Braun Diessel Biotech) instrument.

Protein content was measured with the Folin phenol reagent (Lowry et al., 1951).

SDS-PAGE

Vertical SDS-PAGE and the fixation of proteins after electrophoresis were done as described in a previous paper (Bernáth et al., 1997). Sample volume was 50 µl, which contained 25 µg protein. The correlation of molecular mass and running distance was studied by running a calibration mixture (Pharmacia LMW Kit), and the results were processed by regression analysis. After electrophoresis and fixation, the protein fractions were detected by Coomassie brilliant blue R-250 staining.

Evaluation of the results

The gel electropherograms were digitised with the help of a scanner. Digitised scans were evaluated by the Quantiscan for Windows (Biosoft, Cambridge, UK) program. The running lanes of the individual strains were marked on the digitised picture of the gels, and densitograms were drawn up with the help of the program. Figure 1 shows the densitogram of the strain designated Kacsa (Duck) 344. Protein fractions are represented by peaks in the graph. The areas under the peaks were considered to be proportional to the amount of protein in the fraction. The total amount of electrophoresed protein is represented by the entire area under the curve in the graph.

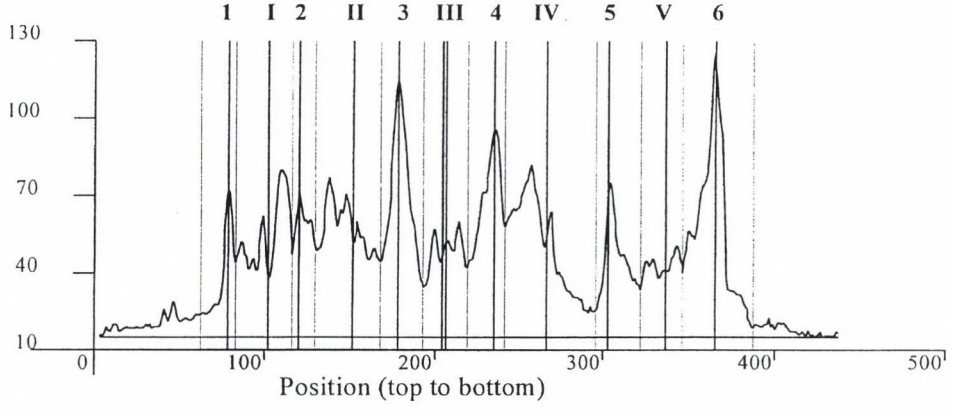

Fig. 1. Densitogram of the SDS-PAGE protein profiles of the *Erysipelothrix rhusiopathiae* strain designated Kacsa (Duck) 344. Protein peaks 1–6 and areas I–V between the peaks are divided within the protein profile to facilitate calculation of the protein distribution

Six peaks were selected, which corresponded to protein fractions present in all the strains examined. The molecular mass of these proteins was 87.4, 66.5, 45.2, 30.9, 19.5 and 13.0 kDa. Subsequently, the protein quantity represented by the above-mentioned six peaks and the five areas flanked by them was calculated

for all strains as a percentage of the entire electrophoresed protein quantity of the strain (entire area under the densitogram). For the calculation, the entire area under the curve as well as the area under each of the 11 segments of the curve were determined with the help of the computer program, by integration. In that way the protein patterns of each bacterial strain were characterised by 11 numerical values. From these 11 values, Pearson's correlation coefficient was calculated using the Statistica for Windows 4.5 (Statsoft Inc., 1993) program. That coefficient characterised the degree of similarity between two strains (Johnson and Wichern, 1982) when pairs of strains were compared for their SDS-PAGE protein patterns. The values of correlation constants were given after multiplication by 1000. In that representation, the maximum value of the correlation coefficient is 1000, which corresponds to a complete identity, while for entirely different data sets the value is 0.

Results

Figure 2 shows the SDS-PAGE profiles of *E. rhusiopathiae* subtype 1a strains isolated from 3 duck carcasses in the same flock and from 8 pigs. Each strain has about 30 protein fractions. The computer program enabled the graphic illustration of the protein profiles of individual strains.

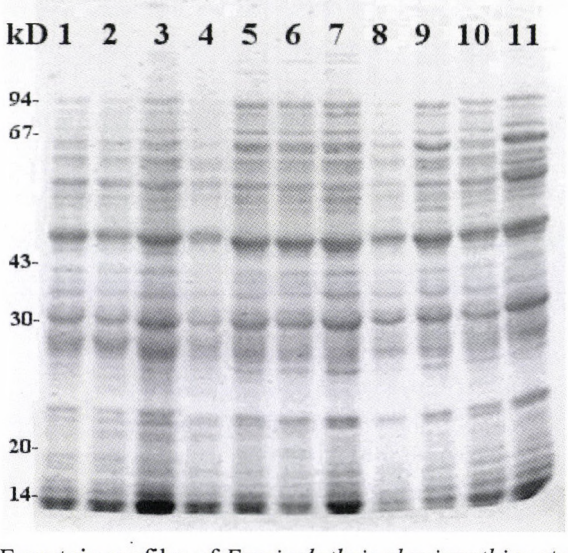

Fig. 2. SDS-PAGE protein profiles of *Erysipelothrix rhusiopathiae* strains isolated from ducks and pigs. Designation of strains: 1: Kacsa (Duck) 509/1; 2: Kacsa (Duck) 397; 3: Kacsa (Duck) 344; 4: Mezőcsát; 5: TU; 6: Bp. XIV; 7: Üllő; 8: Dánszentmiklós; 9: Gyúró; 10: Gyál; 11: A 360

Figure 1 presents a graph illustrating the protein profile of the strain designated Kacsa (Duck) 344. The protein content of the six selected protein fractions (1–6) represented 3.68, 5.04, 11.33, 9.34, 6.44 and 14.11% of the total amount of electrophoresed protein, respectively. The protein content of the five areas (I–V) between the peaks was 10.18, 12.46, 6.83, 15.25 and 5.48%, respectively.

For the three strains isolated from duck carcasses, the value of Pearson's correlation coefficient was between 980 and 991 (Table 1), indicating a high degree of homology.

Table 1

Comparison of the protein composition of *Erysipelothrix rhusiopathiae* subtype 1a strains isolated from ducks

Designation of strains	Similarity*
Kacsa (Duck) 509/1- Kacsa (Duck) 397	984
Kacsa (Duck) 509/1- Kacsa (Duck) 344	980
Kacsa (Duck) 344- Kacsa (Duck) 397	991

*similarity is expressed on a scale of 0 to 1000

When comparing the eight strains isolated from pigs, the extreme values of similarity of the protein fractions were 840 and 989.

The ratio of proteins of identical molecular mass, detected when comparing bacterial strains isolated from ducks and pigs, is given in Table 2. Of the eight strains isolated from pigs, strains designated Mezőcsát and Gyál had a protein composition highly similar to that of the strains of duck origin. At the same time, strains designated Bp. XIV, Gyúró and A 360 showed the highest degree of difference from the strains isolated from ducks.

Table 2

Similarity* of the protein composition of *E. rhusiopathiae* subtype 1a strains isolated from ducks and pigs

Strains isolated from pigs	Strains isolated from ducks		
	Kacsa (Duck) 509/1	Kacsa (Duck) 397	Kacsa (Duck) 344
Mezőcsát	983	990	991
TU	964	939	928
Bp XIV	908	848	836
Üllő	969	962	974
Dánszentmiklós	976	935	933
Gyúró	923	860	850
Gyál	989	979	988
A 360	896	856	876

*similarity is expressed on a scale of 0 to 1000

The reproducibility of the applied methods was checked by the repeated testing of strain A 360. In five simultaneous tests commencing with the culturing of the bacterial strain, the value of similarity of the correlation coefficients was 977 ± 18.

Discussion

Earlier the protein composition of *E. rhusiopathiae* strains was studied by internal radiolabelling of the proteins, SDS-PAGE and autoradiography (Bernáth et al., 1997). The autoradiographic protein profiles of individual strains showed well-visible differences manifesting themselves in the absence or rather in the dissimilar intensity of specific fractions. In the present study, the Coomassie blue staining was used for the detection of proteins. It was found that with the naked eye it was difficult to precisely evaluate the strain-specific differences in the stained protein profiles.

The difference found between the two methods arises from the fact that autoradiography detects only those proteins into which the radiolabelled amino acid was incorporated during the period of culturing. In contrast, the staining procedure can detect all proteins of the bacterial cell. However, the autoradiographic method is not feasible for epidemiological investigation because it takes a very long time to perform. On the other hand, the Coomassie brilliant blue staining procedure is highly suitable also for that purpose, as it takes only about 24 h even in combination with electrophoresis.

For the objective evaluation of the stained protein profiles, we looked for a method by which the similarity or difference of the molecular mass composition of proteins of the bacterial strains could be determined in a precise manner. Computerised evaluation of the densitograms was the method most closely meeting that requirement. As far as we know, a computerised method has not been used yet for evaluating and comparing the SDS-PAGE profiles of *E. rhusiopathiae* strains.

The results obtained for the strains isolated from duck carcasses indicate that these three strains are highly similar to one another. This is explained by the fact that the strains had been isolated from the same duck flock.

The comparison of strains originating from ducks and pigs showed a substantial variability in the molecular mass composition of their proteins. The degree of homology between the strains was between 836 and 991. The results obtained on the similarity or difference of duck strains exhibiting a highly similar protein composition and of the strains isolated from pigs were consistent (Table 2). That finding confirms the reliability of the testing method.

The results suggest that the comparison of the protein patterns of *E. rhusiopathiae* strains provides data that can be used effectively in epidemiological

investigations. The method described in this paper can be a useful complement to procedures recommended for this purpose earlier, i.e. serotyping and multilocus enzyme electrophoresis of the strains.

Acknowledgements

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BACTERIOLOGICAL INVESTIGATIONS ON WOUND MYIASIS OF SHEEP CAUSED BY *WOHLFAHRTIA MAGNIFICA* (DIPTERA: SARCOPHAGIDAE)

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The aim of the present investigation was to get further information about obligate aerobic and facultatively anaerobic bacterial communities of the intact and *Wohlfahrtia magnifica* infested vulval region of sheep. The numbers of aerobic and facultatively anaerobic microorganisms were lower in samples taken from uninfested mucous membrane and myiatic wounds as well as in the wound fluid as compared to samples originating from the uninfested skin surface. Gram-negative bacteria belonging to the family Enterobacteriaceae were isolated only from the skin and mucous membrane of uninfested sheep. Gram-positive microorganisms dominated in all samples. The ratio of facultatively anaerobic bacteria was higher than 80% in the sample taken from a lesion containing third instar *Wohlfahrtia* larvae and in the wound discharge collected from a vulval wound free of maggots. It is suggested that there is a shift in the composition of the bacterial communities of vulva as staphylococci disappear from the wounds due to the presence of *Wohlfahrtia* larvae.

Key words: Myiasis, bacterial communities, *Wohlfahrtia magnifica*

Traumatic myiasis caused by fly larvae is responsible for serious animal health and economic losses in livestock all over the world. Diseases caused by *Lucilia cuprina* and *Cochliomyia hominivorax* (Diptera: Calliphoridae) have been studied in detail (Merritt and Watts, 1978; Dalwitz et al., 1984; Hall and Wall, 1995; Norris, 1995). At the same time, there is scant information about wohlfahrtiosis caused by *Wohlfahrtia magnifica* (Diptera: Sarcophagidae), although this fly species is the main agent of myiasis in many countries of Eurasia (Hall and Wall, 1995; Farkas, 1996). In Hungary, up to 40% of sheep flocks suffer from myiasis at a given time during the summer (Hall et al., 1995; Farkas et al., 1997).

Studies have shown that Wohlfahrtia myiasis may occur in several animal species, mainly in sheep, and it is localised more frequently on undamaged external genital organs such as the vulval region of ewes and the prepuce of rams than in other body regions (Martinez et al., 1987; Nedelchev, 1988; Farkas et al.,

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1997). It is unknown what kind of odours attract gravid female flies to seemingly unhurt parts of the body and help their larviposition. The origin of these semiochemicals is unknown as well. According to the results of studies on *Lucilia* spp. it can be supposed that volatile products of microorganisms living on the skin surface and mucous membranes of sheep can be responsible for fly attractivity and exertion of ovi- and larvipositional responses (Dalwitz et al., 1984).

In order to understand the pathogenesis of wohlfahrtiosis, detailed bacteriological investigations have been carried out.

Materials and methods

Four samples were taken aseptically from four sheep, from areas of the same size (25 cm^2), with cotton swabs moistened in 0.05 M phosphate buffer solution (0.025M Na_2HPO_4 and 0.025M KH_2PO_4 containing 1% TWEEN 80) and 2.5 ml wound discharge was obtained with a sterile syringe. The sampling sites were the following: skin surface and mucous membrane of the vulva of an uninfested sheep, and vulval wounds which contained first and second or third stage larvae of *W. magnifica*, respectively. The fluid was collected from a myiatic wound free of larvae, located in a vulva.

The samples were immediately cooled (4 °C) and transported to the laboratory where they were diluted and plated onto nutrient and King B agar media (King et al., 1954). These media were selected according to the methods described in a previous study (Khoga, 1994) in which it was observed that bacteria originating from wohlfahrtiosis showed the best growth on them. Colony Forming Unit (CFU) estimations were made after incubation for a week at 35 °C. Subsequently, 768 colonies representing the samples were isolated non-selectively, and grouped by their origin and colony-morphological properties. Finally 169 representative isolates were selected from the groups, based on the numerical ratio, for further investigations.

The selected isolates were purified and subjected to the following phenotypic characterisations: Gram staining from 24-, 48- and 72-hour cultures (Claus, 1992), spore staining (Gerhardt et al., 1994), oxidation-fermentation test of glucose (Hugh and Leifson, 1953), oxidase and catalase production (Gerhardt et al., 1994), pigment production on media containing egg-white, and motility in native preparations. The 'metabolic fingerprint' of the strains was tested with the help of Biolog System (Biolog Incorporated, Hayward, CA), studying the utilisation of 95 different carbon sources. The Biolog System was used for genus and occasionally species level identification and cluster analysis (Miller, 1991). The detection of the basic cell-wall components (the diamino-pimelic-acid content (DAP) and the whole-cell-sugar pattern) from whole cell lysates using thin layer

chromatography also supported the precise identification of the phenons (Hasegawa and Takizawa, 1983). Identification was also facilitated by the following phenotypic tests: detection of NaCl and pH tolerances in peptone broth; oxidation-reduction test of mannose, raffinose and galactose in Hugh and Leifson's medium (Hugh and Leifson, 1953), hydrolysis of starch and urea, and detection of phosphatase activity. The key characteristics of Bergey's Manual of Systematic Bacteriology were used for the identification of the following taxa: Enterobacteriaceae (Brenner, 1986), *Staphylococcus* (Kloos and Schleifer, 1986), *Corynebacterium* (Collins and Cummins, 1986), *Bacillus* (Claus and Berkeley, 1986), *Microbacterium* (Collins and Keddie, 1986), *Micrococcus* (Kocur, 1986), *Brevibacterium* (Jones and Keddie, 1986).

Results

Characteristic differences were observed among the samples in the numbers of Colony Forming Units (CFU), but the values of individual samples did not show significant differences between the two media (Fig. 1). The highest values ($470 \times 10^3/25$ cm^2 and $520 \times 10^3/25$ cm^2) were estimated for the sample taken from the skin surface of an uninfested sheep. The CFU numbers were at least tenfold lower ($44 \times 10^3/25$ cm^2 and $23 \times 10^3/25$ cm^2) in the sample originating from the mucous membrane of the same animal. With respect to the myiatic wounds, higher CFU values were obtained for those in which older *Wohlfahrtia* larvae were developing ($150 \times 10^3/25$ cm^2 and $250 \times 10^3/25$ cm^2). In the wound discharge fluid only a few bacteria ($0.02 \times 10^3/2.5$ ml and $0.012 \times 10^3/2.5$ ml) were detected.

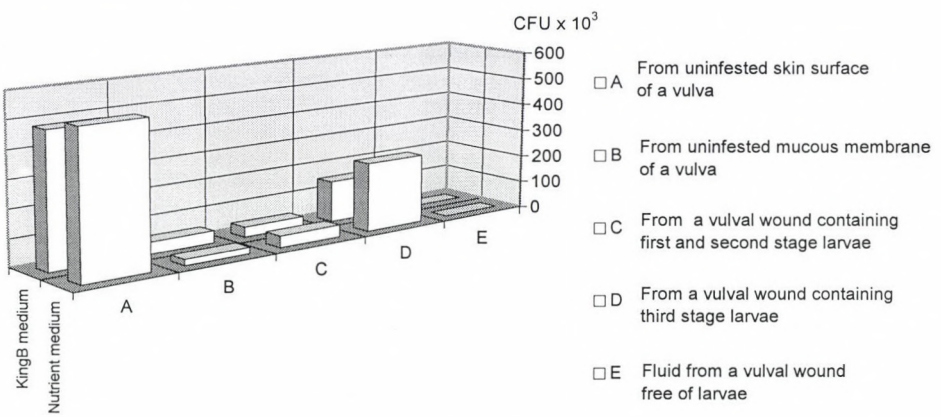

Fig. 1. Colony Forming Units of aerobic and facultatively anaerobic bacterial communities of the samples taken from uninfested and *W. magnifica* infested sheep

Gram-negative bacteria belonging to the family Enterobacteriaceae were isolated only from the skin and mucous membrane of uninfested sheep. These bacteria did not occur in the samples taken from wounds infested with *Wohlfahrtia* larvae.

Gram-positive microorganisms dominated in all samples. The results of identification of Gram-positive bacterial communities are depicted in the form of dendrograms (Figs 2 and 3). The percentage ratio of the genera occurring in the samples is summarized in Table 1.

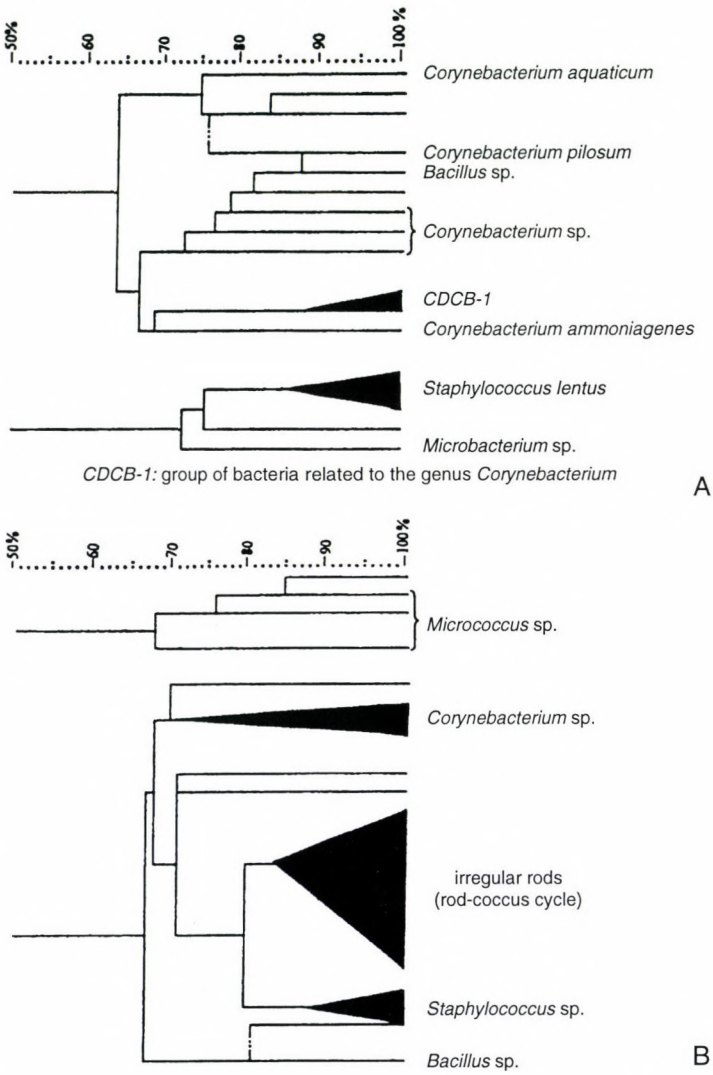

Fig. 2. Gram-positive bacterial communities of healthy vulval regions of sheep. (A) Healthy skin surface of vulva; (B) Healthy mucous membrane of vulva

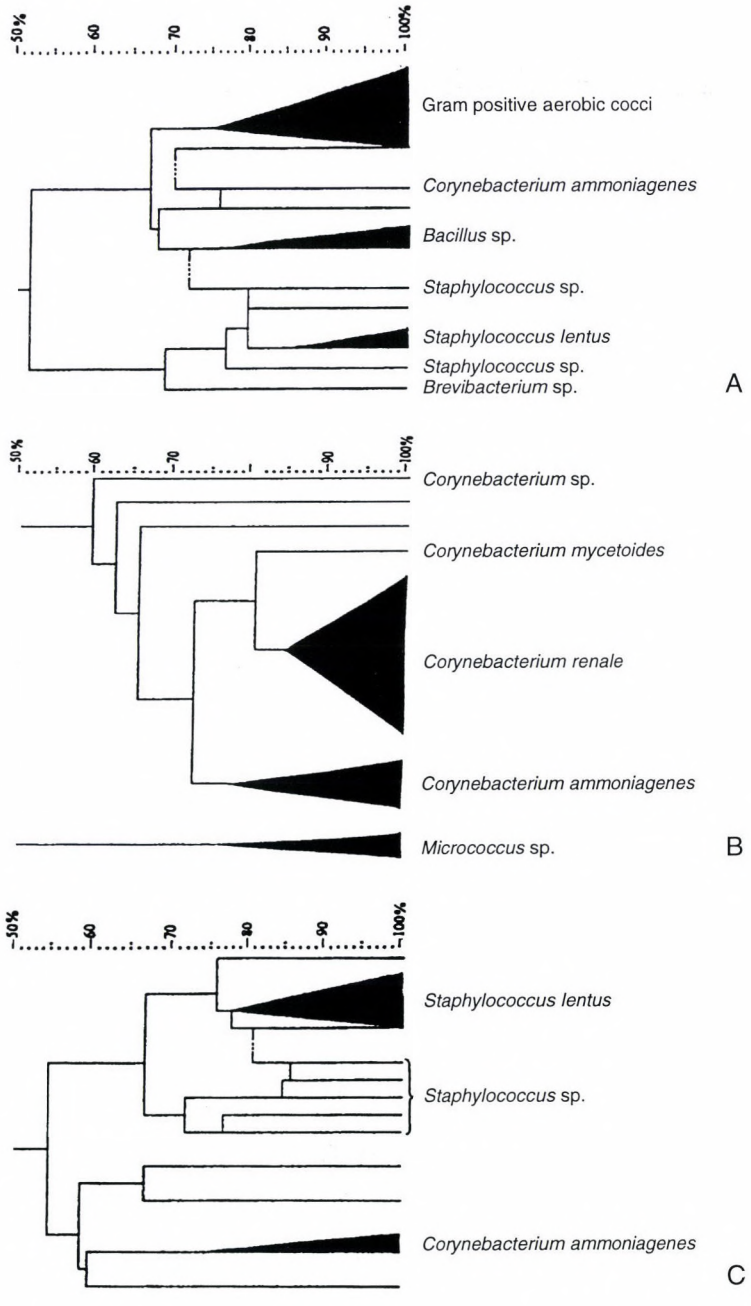

Fig. 3. Gram-positive bacterial communities of myiatic vulval regions of sheep (A) Wound of vulva containing fly larvae of the 1st and 2nd stage; (B) Wound of vulva containing fly larvae of the 3rd stage; (C) Wound discharge

On the skin surface of the uninfested animal the dominance of different *Corynebacterium* species was characteristic, while *Bacillus* and *Microbacterium* species as well as *Staphylococcus lentus* were also present (Fig. 2/A, Table 1).

In the sample originating from the mucous membrane of an uninfested vulva (Fig. 2/B, Table 1) more than 50% of the bacteria could not be identified by the methods applied, however, they were most probably related to the *'Brevibacterium-Arthrobacter'* group of coryneforms. Some *Corynebacterium*, *Staphylococcus, Bacillus* and *Micrococcus* species were also found in this sample.

One-third of the bacteria isolated from the wound containing young (first and second instar) larvae of *W. magnifica* were unidentified Gram-positive aerobic cocci (Table 1). *Staphylococcus*, *Bacillus* and *Brevibacterium* species as well as *Corynebacterium ammoniagenes* were also identified in that sample (Fig. 3/A).

Non-pathogenic *Corynebacterium* species, mainly *C. renale* dominated in the sample collected from the vulval wound containing third stage larvae (Table 1), but *Micrococcus* species were also present (Fig. 3/B).

In the wound discharge, members of the genus *Staphylococcus* were present in the highest quantity (Table 1), but *C. ammoniagenes* was also found in it (Fig. 3/C).

Table 1

The percentage ratio of Gram-positive bacterial genera in the samples

Genera	Samples taken from				
	uninfested skin surface of a vulva	uninfested mucous membrane of a vulva	wound of a vulva containing first and second stage larvae	wound of a vulva containing third stage larvae	a vulval wound free of larvae
Corynebacterium	41.0	10.5	6.3	76.4	14.3
Bacillus	6.7	5.2	12.4	0.0	0.0
Staphylococcus	21.0	15.8	31.3	0.0	57.2
Micrococcus	0.0	15.8	0.0	11.8	0.0
Microbacterium	6.7	0.0	0.0	0.0	0.0
Brevibacterium	0.0	0.0	6.3	0.0	0.0
Unidentified bacteria	24.6	52.7[(1)]	43.7[(2)]	11.8	28.5

[(1)] These bacteria were mainly irregular rods having morphological cell-cycle. They were most related to the *'Brevibacterium-Arthrobacter'* group of coryneforms; [(2)] 31% of the bacteria found (aerobic cocci in Fig. 3/A) were most related to the genus *Micrococcus*

The ratio of facultatively anaerobic bacteria was higher than 80% in two of the five samples (Fig. 4).

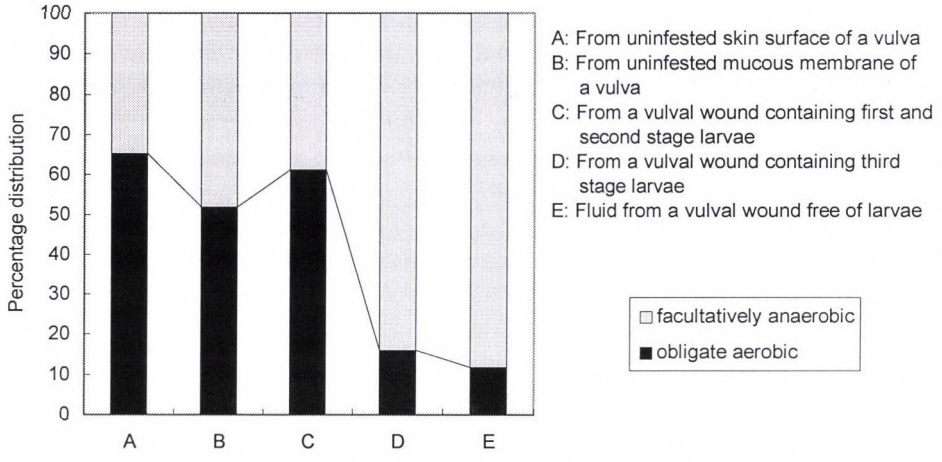

Fig. 4. Ratio of obligate aerobic and facultatively anaerobic bacterial strains in the samples

Discussion

Several laboratory and field studies have demonstrated that odours are very important factors in long-distance orientation, alighting and oviposition responses of myiasis-causing *Lucilia* species (Emmens and Murray, 1982, 1983; Ashworth and Wall, 1994; Morris et al., 1997). As regards the sources of these odours, it can be concluded that volatile substances are produced by the action of microorganisms in wounds, skin lesions and fleece. It is supposed that odours originating from various sources may also have a key role in host location, landing and larviposition of *Wohlfahrtia* females. For this reason, the detailed microbiological investigation of wohlfahrtiosis is also very important. The first bacteriological studies on *Wohlfahrtia* myiasis were carried out by Khoga (1994). The aim of the present investigation was to obtain further basic information on the bacterial communities of the intact and *W. magnifica* infested vulval region of sheep.

Studying the CFU numbers of aerobic and facultatively anaerobic strains, it could be concluded that they were lower in samples taken from uninfested mucous membrane and myiatic wounds as well as in the wound fluid (Fig. 1). Similar results were obtained by Khoga (1994), and other researchers also found fewer bacteria on mucous membranes than on the skin surface of healthy animals (Webster et al., 1981). There are some possible explanations of the lower CFU values of myiatic lesions including the wound fluid. It cannot be ruled out that the fly larvae consumed a large amount of the bacteria and/or that the multiplication of these microorganisms was inhibited by antimicrobial compounds

produced by larvae or their bacterial communities (Erdman et al., 1984). These data could also be connected with the development of *Wohlfahrtia* larvae. The larvae gradually pad the wounds and for this reason anaerobic conditions might dominate there. This is supported by the ratio of obligate aerobic and facultatively anaerobic microbial communities found in the samples (Fig. 4).

The dominance of Gram-positive microorganisms in all samples corresponds to the data of Khoga (1994). He also observed that microorganisms belonging to the family Enterobacteriaceae disappeared from the wounds due to wohlfahrtiosis; however, in contrast to our results he found some other Gram-negative bacteria (e.g. *Pseudomonas* spp.). There were characteristic differences among the samples in the Gram-positive microorganisms present (Figs 2 and 3, Table 1). The presence of *Staphylococcus* and *Corynebacterium* species in samples taken from the skin and mucous membrane of uninfested sheep is not unusual because they were found to be typical members of the microbial communities of epithelial surfaces in warm-blooded animals (Collins and Cummins, 1986; Kloos and Schleifer, 1986). *S. lentus,* the organism identified at species level, has been isolated only from scarfskins of sheep and from udder of goat so far (Kloos and Schleifer, 1986). Bacteria having typical morphological cell-cycle (irregular rods) were found in a relatively high amount in the sample originating from the mucous membrane of uninfested vulva (Fig. 2/B, Table 1). They were most related to the *'Brevibacterium-Arthrobacter'* group of coryneforms, though these bacteria could not be identified precisely by the methods applied. *Brevibacterium* species are well known for the production of malodorous volatile compounds (Sharpe et al., 1977). Khoga (1994) also found these microorganisms and observed in his field studies that the volatile chemicals (mainly thiol compounds) produced by them attracted many *W. magnifica* females to the traps. Based on these results it can be supposed that *Brevibacterium* species may play an important role in the infestation of undamaged mucous membranes of sheep by *W. magnifica.*

Purulence could not be observed in any case of wohlfahrtiosis when samples were collected, despite the fact that some pyogenic cocci were present in the wound containing young larvae (Fig. 3/A). This clinical symptom, which is a common observation (Farkas et al., 1997), could be associated with *Wohlfahrtia* maggots because the members of the genus *Staphylococcus* completely disappeared from the wound filled with third instar larvae. Non-purulence of the wounds infested with *C. hominivorax* larvae was explained by the antimicrobial compounds produced by *Proteus mirabilis* found in the intestine of the maggots (Erdman et al., 1984). Staphylococci, which are facultatively pathogenic and pyogenic microorganisms (Kloos and Schleifer, 1986), were found in a relatively high ratio (Fig. 3/C, Table 1) in the fluid collected from the wound free of maggots. In this case the bacteria, mainly the pyogenic species, could get into

the wounds from the surrounding surfaces. Later these microorganisms can multiply due to the lack of the antibacterial effects of fly larvae and/or their microbial communities. For this reason suppuration may occur in the wounds free of larvae (Farkas et al., 1997).

Corynebacterium species (*C. ammoniagenes* and *C. renale*) were dominant in the wound containing older *Wohlfahrtia* larvae. These opportunistic microorganisms could get into the wounds from the urine because they are common representatives of the autochtonous microbial communities of the urinary tract of sheep (Collins and Cummins, 1986).

The facultatively anaerobic members of the genera *Corynebacterium* or *Staphylococcus* were characteristic in the sample taken from the vulval wound filled with third instar larvae and in the wound discharge (Fig. 4). It can be supposed that anaerobic conditions started to become dominant in these lesions. Detailed studies should be done to get information on the composition of obligate anaerobic microbial communities of this type of wounds.

Further bacteriological studies are needed to identify the volatile substances responsible for attracting gravid females to undamaged and myiatic external genitalia or other natural body orifices and for their larviposition in wohlfahrtiosis. The other main goal of such investigations is to detect the sources of these chemicals. In addition to giving a closer insight into the behaviour of *W. magnifica* and the pathogenesis of the disease, these results might also be useful in the development of a species-specific synthetic attractant as an element of alternative control techniques rendered necessary by environmental as well as animal and public health concerns.

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EFFECTS OF PHYTASE SUPPLEMENTATION ON CALCIUM AND PHOSPHORUS OUTPUT, PRODUCTION TRAITS AND MECHANICAL STABILITY OF THE TIBIA IN BROILER CHICKENS

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A feeding trial was performed using 4 × 60 day-old chickens (Ross 208 cockerels) raised up to 42 days of age to determine whether exogenous phytase addition increases phosphorus utilisation by broiler chickens, and to assess its effects on some production traits as well as on the ash content and mechanical stability of the tibia. The chickens' feed consisted of maize, wheat, soybean meal, fish meal, yeast, and fat powder. The basic feed was supplemented with inorganic phosphorus in groups A and B. In groups C and D, the amount of the inorganic phosphorus supplement (DCP) was decreased by 50%, at the same calcium/phosphorus ratio. The 50% reduction of inorganic phosphorus supplementation represents a 20% decrease of total phosphorus. To the diets of groups B and D a phytase enzyme preparation (Phytase Novo CT™) was added. The calculated exogenous phytase activity was 600 FYT/kg feed. The decrease of inorganic phosphorus did not cause significant differences in the daily weight gain but lowered the feed conversion rate by 10%. Calcium and phosphorus excretion decreased by 18% and 15%, and the breaking strength of the tibia was also lower. Phytase supplementation of the feed at a lower rate of inorganic phosphorus supplementation did not cause changes in the body weight gain but improved the feed conversion rate by 5.6%. Phosphorus and calcium output decreased by 21% and 11%, respectively, but chemical composition and mechanical stability of the tibia were unaltered.

Key words: Phytase, phosphorus, calcium, tibia, mechanical stability, broiler chickens

Most cereal feedstuffs are characterised by a low (approx. 30–35%) availability of phosphorus for monogastric animals. This is because two-thirds of their phosphorus content is in phytate form which is largely unavailable to birds, since they lack the phytase enzyme necessary for hydrolysing phytate. This is the main reason why inorganic phosphorus supplementation is used in monogastric diets to meet phosphorus requirements. The majority of phytic phos-

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phorus passes into the faeces in non-digested form and causes an increased environmental load (Khan, 1995).

There is abundant experimental evidence that supplementation of the diet of monogastric animals with exogenous phytase enzyme of fungal origin without inorganic phosphate supplementation increases the apparent digestibility of plant phosphorus (Simons et al., 1990; Beers and Jongbloed, 1992; Cole and Khan, 1994), calcium (Halle et al., 1996; Huyghebaert, 1996*b*), magnesium (Pallauf et al., 1992) and even iron and zinc (Gebert and Wenk, 1995).

Higher daily weight gain (Cantor and Perney, 1992*)* and higher nitrogen retention in broiler chickens (Christensen et al., 1996*)* and higher daily weight gain in growing pigs (Poulsen, 1993; Khan and Cole, 1995) have also been found.

In addition to decreasing or eliminating the need for inorganic phosphorus supplementation, the supplementation of poultry feeds with microbial phytase reduces the environmental load.

The objective of the present study was to study the effect exerted by a lowered dietary level of inorganic phosphorus, with or without phytase enzyme addition, on some production traits, phosphorus and calcium excretion as well as on the ash content and breaking strength of the tibia in broiler chickens.

Materials ad methods

Birds and diets

A model feeding experiment was carried out with broiler chickens (Ross 208 hybrid cockerels). Each treatment group included 6 × 10 birds. The chickens were kept in four-tier cages according to the generally accepted technology. They were fed a started diet from day old to 21 days of age, a grower diet from 22 to 35 days of age, and a finisher diet between 36 and 42 days of age *ad libitum*. All the diets were ground but not pelleted.

Two types of ration were formulated: the first type (diets A and B) was the control diet with a nutrient content according to Hungarian standards (*control diet*); the second type (diets C and D) was the same as the control except that the inorganic phosphorus supplement (DCP) was decreased by 50% at the same Ca/P ratio (*low-phosphorus diet*).

A phytase enzyme preparation (Phytase Novo CT™, Novo Nordisk A/S, Copenhagen) was used in a dose of 0.24 kg/t feed in diets B and D, which meant 600 FYT units/kg of feed.

Experimental design:

Treatment	Diet	Number of birds
A	control	6 × 10
B	control + phytase	6 × 10
C	low phosphorus	6 × 10
D	low phosphorus + phytase	6 × 10

Methods of measurement

The nutrient content as well as the calcium and phosphorus content of the feeds were determined (Table 1) according to the Hungarian National Standard method (Hungarian Feed Codex, 1990).

The excreta were collected from each group and their quantity as well as dry matter, phosphorus and calcium contents were determined weekly. The average daily P and Ca outputs were calculated.

The dry matter, ash, calcium and phosphorus contents of both tibia bones of two chickens in each group (6 × 2 = 12/treatment) were determined according to the Hungarian National Standard Method (Hungarian Feed Codex, 1990).

During the experiment the daily feed intake per group and the individual body weight were determined at the end of each phase, so that the average weight gain and feed conversion rate could be calculated.

At 42 days of age 5 randomly selected chickens were slaughtered from each treatment group and their grill weights were measured.

Measurement of the mechanical stability of tibia

The tibia (anatomically the tibiotarsus) of both legs was removed from 3 chickens of each group (6 × 3 = 18/treatment) and its mechanical stability was determined. The outer diameter and, after breaking the bones, the inner diameter were measured at two different points (which were, however, at exactly the same location on each bone) and the thickness of the bone wall was calculated. The tibia bones of the right legs were broken by exerting on them a force of axial direction. The breaking strength and the average diameter were used to calculate the compressive strength. The tibia bones of the left legs were broken using three-point shoring. The ratio of breaking strength and average diameter was used to calculate the bending strength. The measurements were performed according to the method of Gelencsér and Csermely (1981).

Statistical analysis

The statistical analysis of data was done by Student's *t*-test.

Table 1

Composition and nutrient content of broiler diets

	Starter		Grower		Finisher	
	control	low P	control	low P	control	low P
Maize, %	48.3	49.5	53.4	54.7	55.2	56.0
Wheat, %	20.0	20.0	20.0	20.0	20.0	20.0
E. soybean, 46%	22.5	22.5	20.5	20.5	21.5	21.5
Fish meal, 64%	4.0	4.0	2.0	2.0	–	–
Fat powder, %	1.3	0.9	0.5	–	–	–
AP 17 (DCP), %	1.6	0.8	1.3	0.5	1.5	0.7
Limestone, %	1.1	1.1	0.9	0.9	1.0	1.0
Biomethine, 20%	0.4	0.4	0.3	0.3	–	–
Biolysine, 20%	0.1	0.1	0.4	0.4	–	–
NaCl, %	0.2	0.2	0.2	0.2	0.3	0.3
Vitamin and trace mineral premix, %	0.5	0.5	0.5	0.5	0.5	0.5
Total, %	100.0	100.0	100.0	100.0	100.0	100.0
	Calculated nutrient content					
AME, MJ/kg	12.4	12.4	12.4	12.4	12.4	12.4
Methionine, g/kg	4.5	4.5	3.8	3.8	3.0	3.0
Methionine + Cysteine, g/kg	7.4	7.5	6.6	6.6	5.7	5.7
Lysine, g/kg	11.0	11.0	10.1	10.1	8.7	8.7
Crude protein/ME g/MJ	15.9	15.9	14.4	14.4	13.9	13.8
	Analysed nutrient content					
Dry matter, g	900.0	900.0	900.0	900.0	900.0	900.0
Crude protein, g	196.0	194.0	175.5	175.0	169.0	170.0
Crude fat, g	35.0	35.0	32.2	30.5	30.0	29.6
Crude fibre, g	27.0	26.5	31.1	31.0	32.4	31.2
Ash, g	55.0	44.0	54.1	33.8	53.2	39.8
Ca, g	11.1	8.9	7.9	6.2	7.9	7.0
P (total), g	6.9	5.7	5.8	4.4	5.8	5.0
Ca /P ratio	1.6	1.6	1.4	1.4	1.4	1.4

Results and discussion

Production traits

Phytase supplementation of the control diet (B) did not have a significant effect on the chickens' daily weight gain (Table 2). The moderately lower average values obtained during the first and second phase and the moderate increase

found during the third phase of growth were not statistically significant. That result was similar to the findings of a previous experiment (Huyghebaert, 1996*a*). The lower total phosphorus content of diet C decreased the daily weight gain during the whole growing period as compared to the control where this occurred mainly in the growing and finishing periods (Table 2). The difference in average liveweight was statistically significant at 35 and 42 days of age. An opposite result was obtained in a more recent investigation (Guo et al., 1996), where a diet of lowered calcium and phosphorus content did not affect the production traits during the finishing phase of growing. It is obvious that the difference between the two experiments was due to the dissimilar amounts deposited of these two elements.

Table 2

Effect of phytase supplementation on the production traits of broilers

Production traits	Treatment			
	control A	control + phytase B	low P C	low P + phytase D
Number of chickens	60	60	60	60
Liveweight of day-old chickens, g	42	42	41	41
Day old to 21 days of age:				
Liveweight at 21 days, g	559±60 NS	555±63 NS	550±61 NS	572±60 NS
Average weight gain, g	517	513	509	531
Feed conversion rate, kg/kg	1.73	1.74	1.74	1.68
Mortality (n)	0	0	0	0
21–35 days of age:				
Liveweight at 35 days, g	1307±134	1286±137	1252±145[a]	1269±134
Average weight gain, g	748	731	702	697
Feed conversion rate, kg/kg	2.17	2.31	2.42	2.42
Mortality (n)	1	1	0	1
36–42 days of age:				
Liveweight at 42 days, g	1672±188[c]	1665±185 NS	1539±256[a, d]	1616±164[c]
Average weight gain, g	365	379	287	347
Feed conversion rate, kg/kg	3.41	3.27	4.29	3.64
Mortality (n)	0	0	0	0
Day old to 42 days of age:				
Average weight gain, g	1630	1623	1498	1575
Average daily weight gain, g	38.8	38.6	35.7	37.5
Feed conversion rate, kg/kg	2.3	2.35	2.54	2.43
Mortality (n)	1	1	0	1

NS = no significant difference ($P > 0.05$); [a–d] = means in the same row bearing different superscripts differ significantly ($P < 0.05$)

Phytase supplementation (b) improved the daily weight gain during the starter and finisher phases, but the difference was significant only within the finisher phase. The daily weight gain of birds in the low-phosphorus + phytase-supplemented group (D) was similar as in the control groups; the differences were not statistically significant.

At the end of the experiment (at 42 days of age) the liveweight was significantly lower in the group fed the low-phosphorus diet (C) without phytase than in the control (A) and in the low-phosphorus + phytase (D) group (Table 2).

The feed conversion rate changed according to the different treatments (Table 2). The moderate increase of feed intake and the above-mentioned moderately lower daily weight gain resulted in a higher absolute feed consumption per kg of body weight gain in the treated groups than in the control.

The cumulative feed conversion ratio was 10% worse (2.54 kg) in group C as compared to the control (2.30 kg), but phytase supplementation brought about a moderate improvement (2.43 kg).

The health status of chickens was optimal throughout the trial, as evidenced also by the negligible mortality rate.

Slaughter value

There was no significant difference in the slaughter value of birds at 42 days of age (Table 3), but the results showed that reduced phosphorus supplementation of the diets decreased not only the final body weight but also the grill weight in group C, while phytase enzyme supplementation caused changes of the opposite tendency in group D.

Table 3

Effect of phytase supplementation on carcass quality

Carcass quality traits	Treatment			
	control a	control + phytase b	low P c	low P + phytase d
Liveweight of slaughtered chickens, g	1669±101 NS	1648±99 NS	1566±109 NS	1617±72 NS
Grill weight, g	1058±66 NS	1045±76 NS	983±78 NS	1012±65 NS
Average grill weight in percent of liveweight, %	64.4	63.4	62.7	62.6

NS = no significant difference

Chemical composition of excreta

No significant difference was found in the dry matter content of excreta (Table 4). Although phytase supplementation caused some statistically significant differences, the changes did not show a consistent tendency. In another recent investigation using phytase supplementation (Jensen et al., 1996), no measurable difference was found in the mortality rate and in the quality of manure either.

Table 4

Effect of phytase supplementation on the dry matter content of excreta, %

Age (days)	Dry matter content of excreta (%)			
	control A	control + phytase B	low P C	low P + phytase D
1–7	21.2±2.3 NS	21.0±1.7 NS	21.6±1.8^{d}	19.7±2.0^{c}
8–14	20.9±1.7^{b}	20.0±1.1^{a}	21.0±1.2^{d}	19.9±1.7^{c}
15–21	20.4±2.9^{b}	19.5±2.2^{a}	20.8±1.7 NS	20.0±2.1 NS
22–28	20.9±2.6 NS	19.8±3.7 NS	21.1±3.0^{d}	19.6±1.4^{c}
29–35	18.6±3.2 NS	19.6±1.6 NS	18.9±3.6 NS	18.7±2.2 NS
36–42	19.1±2.4 NS	19.1±2.2 NS	18.6±2.6 NS	18.2±2.5 NS
Mean	*20.2±2.1 NS*	*19.8±2.2 NS*	*20.3±2.5 NS*	*19.4±1.8 NS*

NS = no significant difference ($P > 0.05$); $^{a–d}$ = means in the same row bearing different superscripts differ significantly ($P < 0.05$)

The faeces was collected regularly from each group and the output of calcium and phosphorus was determined. The average daily phosphorus output (Table 5) suggests that lower dietary phosphorus content (groups C and D) resulted in the absorption of a higher amount of phosphorus throughout the growth period. The decrease in phosphorus output was 31.8% within the starter phase (groups C and D), 23.6% and 28.8% within the grower phase and 19.1% and 4.3% in the finisher phase, respectively. Calculated for the whole growth period, chickens fed less dietary phosphorus (groups C and D) excreted less phosphorus than the control birds. The same result was obtained in another experiment with different genotypes of chicken (Tyagi et al., 1996), and it was suggested that the calcium and phosphorus content of the diet might influence phosphorus retention. Phytase supplementation of the control diet moderately decreased the phosphorus output but the difference was not statistically significant.

Average daily calcium output (Table 6) showed that phytase supplementation of the control diet did not cause appreciable changes. The diet containing

less phosphorus and calcium (C) decreased the calcium output by 25.9%, 21.8% and 6.7% in the starter, grower and finisher phase, respectively. During the starter and grower phases, calcium output decreased by the same percentage in the group fed a low-calcium and low-phosphorus diet supplemented with phytase enzyme (D) as in the group not receiving phytase; however, in the finisher phase calcium output markedly increased in the phytase-supplemented group as compared to the control. It can be established that group D showed a higher absolute phosphorus output during that period of growth (Table 5).

Table 5

Effect of phytase supplementation on average phosphorus output (mg)

Age (days)	Average phosphorus output (mg)			
	control A	control + phytase B	low P C	low P + phytase D
1–7	$14\pm2.1^{c,d}$	12 ± 1.4^{d}	9 ± 0.4^{a}	$7\pm3.3^{a,b}$
8–14	$83\pm13.8^{c,d}$	$71\pm9.1^{c,d}$	$39\pm4.0^{a,b}$	$40\pm5.5^{a,b}$
15–21	$128\pm18.0^{c,d}$	$129\pm17.0^{c,d}$	$95\pm12.0^{a,b}$	$95\pm19.1^{a,b}$
1–21	*88±50.9 NS*	*85±52.0 NS*	*60±39.8 NS*	*60±41.3 NS*
22–28	164 ± 29.9^{d}	149 ± 18.7^{d}	140 ± 17.5^{d}	$115\pm16.2^{a,b,c}$
29–35	218 ± 39.4^{c}	221±21.7 NS	$153\pm12.3^{a,b}$	$157\pm24.0^{a,b}$
22–35	$191\pm43.8^{c,d}$	$181\pm42.8^{c,d}$	$146\pm15.9^{a,b}$	$136\pm29.5^{a,b}$
36–42	230 ± 22.7^{c}	237 ± 27.6^{c}	$186\pm6.2^{a,b}$	220 ± 28.4^{c}
1–42	*155±72.9 NS*	*148±75.9 NS*	*131±48.7 NS*	*122±69.0 NS*

NS = no significant difference ($P > 0.05$); $^{a-d}$ = means in the same row bearing different superscripts differ significantly ($P < 0.05$)

Chemical composition of tibia

The possible difference in calcium and phosphorus deposition and/or incorporation did not have a measurable effect on the chemical composition of bones (Table 7). There were no interpretable differences in the dry matter, ash, calcium and phosphorus content of tibia among the groups. Rao et al. (1996) showed that the tibia of broiler chickens had higher phosphorus content in phytase-supplemented groups fed either diets of normal P content or low-phosphorus diets. The difference was probably due to the different activity of enzymes used.

Table 6

Effect of phytase supplementation on average daily calcium output (mg)

Age (days)	Average daily calcium output (mg)			
	control A	control + phytase B	low P C	low P + phytase D
1–7	51±8.8^{c,d}	51±7.6 NS	34±9.1^{a}	311±4.5^{a}
8–14	228±40.0^{c,d}	202±32.0^{c,d}	101±9.5^{a,b}	118±8.8^{a,b}
15–21	426±52.6^{c}	453±59.8^{c}	333±50.3^{a,b}	349±56.7 NS
1–21	*270±168.8^{c,d}*	*290±184.2^{c,d}*	*200±144.8^{a,b}*	*211±152.0^{a,b}*
22–28	521±62.5 NS	568±84.3^{c}	445±62.7^{b}	470±69.6 NS
29–35	750±126.0 NS	747±82.2^{d}	550±47.9 NS	546±78.1^{b}
22–35	*637±151.8 NS*	*638±124.9 NS*	*498±76.5 NS*	*508±82.3 NS*
36–42	676±83.8^{d}	705±83.3 NS	631±46.2 NS	802±102.9^{a}
1–42	*503±248.5 NS*	*489±196.3 NS*	*411±162.2 NS*	*448±211.0 NS*

NS = no significant difference ($P > 0.05$); $^{a–d}$ = means in the same row bearing different superscripts differ significantly ($P < 0.05$)

Nor were there significant differences among the groups in the calcium and phosphorus content of tibia as calculated in percent of ash, and the values were within the physiological range. The results suggest that the lowered dietary phosphorus level combined with a moderately decreased dietary calcium content did not cause a detectable disturbance in the mineral metabolism of chickens. It can also be stated that phytase supplementation increases the incorporation rate of minerals.

Mechanical stability of tibia

The above statement does not contradict the results obtained on mechanical stability. Phytase supplementation of the control diet (B) did not cause changes in mechanical stability. Feeding the low-phosphorus diet (C) decreased the compressive strength, and the bending strength also decreased (though not significantly) as compared to the control. Addition of phytase to the low-phosphorus diet (D) resulted in the same breaking strength and compressive strength as in the control group, while it increased the bending strength moderately but not significantly (Table 8).

Table 7

Effect of phytase supplementation on the chemical composition of tibia (n = 12)

	Chemical composition of tibia			
	control A	control + phytase B	low P C	low P + phytase D
Original dry matter, %	55.0±1.7 NS	55.0±1.6 NS	56.8±2.1 NS	56.8±2.1 NS
Bone ash, %	38.9±0.9 NS	39.2±1.8 NS	36.4±1.7 NS	37.6±1.6 NS
Ca content, %	14.2±1.6 NS	14.2±1.5 NS	13.2±1.4 NS	13.1±1.0 NS
P content, %	7.5±0.2 NS	7.6±0.4 NS	7.3±0.7 NS	7.2±0.3 NS
Ca in % of bone ash, %	36.6±4.6 NS	36.2±4.6 NS	36.5±4.8 NS	35.0±3.5 NS
P in % of bone ash, %	19.2±0.5 NS	19.2±0.7 NS	20.0±1.0 NS	19.2±0.5 NS

NS = no significant difference ($P > 0.05$)

Table 8

Effect of phytase supplementation on mechanical stability of tibia (n = 18)

Treatment	Compressive strength N/mm^2	Bending strength N/mm^2
A	28.99 ± 7.96 NS	88.50 ± 21.9 NS
B	27.44 ± 6.04 NS	96.51 ± 17.6 NS
C	24.87 ± 3.61^{d}	88.90 ± 21.5 NS
D	28.03 ± 4.39^{c}	98.31 ± 19.5 NS

NS = no significant difference ($P > 0.05$); $^{a-d}$ = means in the same row bearing different superscripts differ significantly ($P < 0.05$)

Conclusions

In agreement with some previous findings (Niekerk and Reuvekamp, 1996), the present study showed that a 50% reduction of the inorganic phosphorus supplementation of broiler chicken diets of generally accepted nutrient content, combined with the addition of a phytase preparation in a dose of 600 FYT units/kg feed, did not cause measurable changes in the production traits (daily weight gain, feed conversion rate, grill weight expressed as percent of live-weight); at the same time, it decreased the environmental load by lowering the calcium and phosphorus output but did not change the calcium and phosphorus content and mechanical stability of the tibia.

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RELATIONSHIP OF DIETARY FAT AND LYSINE LEVEL WITH BODY COMPOSITION IN BROILER CHICKENS

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The effect of dietary fat and/or lysine supplementation on the body composition of broiler chickens was studied. Body composition was measured by computed tomography (CT) and direct chemical analysis. Cockerel chicks from a previous experiment (Fekete et al., 1992) were used in this study. Ten chickens from each treatment group were euthanised, deep frozen and subjected to CT. Subsequently the bodies were dissected and ground to obtain homogeneous samples for chemical analysis. Supplementation of the diet with 6 g/kg lysine did not change total body composition but positively influenced the final body weight. In the treatment group receiving 40 g/kg added fat and 3 g/kg feed lysine (F-LYS-I) the higher body weight ran parallel with a higher fat content. The difference between the frozen body weight and final live body weight shows that lysine supplementation increased the water-holding capacity of muscle, which was the best in groups receiving a diet of normal energy content plus high lysine supplementation.

Key words: Lysine, dietary fat, broiler chicken, body composition, meat quality, computed tomography

The modern market requires a carcass of low fat level. The animal species most prone to fat deposition are the pig and poultry. In poultry, the majority of fat tends to accumulate in the abdominal fat pad. The quantity of deposited fat may vary from 9.5% to 23% in broilers at slaughtering age. Both the quantity and the quality of fat can be modified by several factors including nutrition (Whitehead, 1986). In broilers, fat accumulation is especially fast after 6–7 weeks of age. According to the modern recommendations, broiler chickens should be given a very good energy and protein supply during the rearing period; in this way they can be slaughtered before intensive fat deposition could occur. Since pigs and poultry grow relatively faster than other animals, they require a carefully balanced diet of high energy and amino acid concentration.

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The determination of body composition has great impact on the evaluation of nutrient requirements. Direct chemical analysis is time consuming and expensive. Consequently, several alternative methods and estimations have been developed. Gous (1986) stated that initial and final body weights would be better parameters than the body weight gain (BWG). Selection of birds for determination of body composition should be done at random. Dissection of abdominal fat can be used as an alternative method to determine carcass fat content. The method gives good information on the market value but provides no data on the feed conversion ratio (FCR). Velu et al. (1972*a*) described a linear relationship between body water and body fat content. After the determination of body water content, the equations given by them provide a simple yet reliable means of estimating both body fat content and body protein content in young chickens. The results of Wolynetz and Sibbald (1985) suggest that at the age of 14–18 days the body weight is a good indicator of body composition for male broilers. New methods for the *in vivo* measurement of body composition have acquired increasing importance over the last years. Two of the new methods, computed tomography (CT) and total body electrical conductivity (TOBEC) make it possible to perform the measurement on intact, live animals. These procedures meet not only the nutritional but also the animal welfare demands.

The amino acid requirements expressed in dietary concentration decrease with age due to age-related changes in body composition (Baker, 1989). In broiler nutrition, amino acid requirements depend on several factors including the genotype. This is especially true for lysine, which is the first limiting amino acid in poultry.

Kirchgessner et al. (1988) measured the contents of various amino acids in the whole carcass and in selected broiler organs. The whole carcass, muscle and liver contained 6.5, 8.7 and 7.5% lysine of crude protein, respectively. Gruhn and Zander (1987) found that the digestibility of various amino acids decreased with the increased fibre content of diets. The feed components and their relationships have a great influence upon total body composition.

Amino acid recommendations will be determined by the criteria applied. The publication of Rhône-Poulenc (1988) estimated the lysine requirements if the energy density of the diet was 12.56 MJ ME/kg, 0.95% for starter and 0.80% for finisher; however, it did not state the criteria of evaluation. Target parameters can be maximum carcass leanness, optimum feed conversion ratio, maximum weight gain, meat quality, etc. The requirements for maximum weight gain have the lowest amino acid needs (Sibbald and Wolynetz, 1986; Baker, 1989). Oju et al. (1987) studied the protein, methionine, and lysine requirements of growing turkey hens at various environmental temperatures. Despite the fact that the protein to energy ratio was different in each diet, they found that the protein requirement increased at temperatures higher than 14 °C. The lysine requirement was affected by antagonism (arginine) as well as availability (Maillard reaction).

Lysine is the precursor of carnitine. This compound plays an important role in the transport of fatty acids through mitochondrial membranes. By this, carnitine affects the energy production of all cells of the body, and indirectly influences lipid metabolism by removing end-products from the blood. Carnitine is therefore especially important in the fat metabolism of muscle. High lysine intake may reduce fat synthesis either by increasing the carnitine supply or by stimulating insulin secretion (Rebouche, 1992).

The nutrient requirements of broilers should be given in great detail, as otherwise the diet can cause some unexpected effects. Leanness is an economic target in the poultry industry. This means that the benefits of leanness should be evaluated by looking at the profits. In several trials, leanness was generally associated with an improved FCR. Whitehead (1991) suggested that the rate of lipid uptake by adipose tissue might be an important factor in the birds' fatness. There are genetically different responses to dietary protein, vitamins, and minerals. Therefore, it should be taken into consideration that the genotype can cause as great differences as nutritional treatment. The right approach to leanness is the combination of genetic and nutritional methods. The genetic approach tends to be more economical, while the nutritional one is more rapid.

While studying the effects of temporary amino acid restriction on growth and carcass quality in turkeys, Holsheimer and Ruesink (1993) found that amino acid supplementation over the NRC standards (1984) had a positive effect on feed conversion, growth and carcass quality. Potter and McCarthy (1985) concluded that at an older age turkeys had a better feed conversion ratio if extra fat was added to the diet. Birds consumed more of the concentrated diet, and they gained weight more rapidly. The above-cited authors found that sex had a great influence on the effect of amino acids. This sex-related difference in the response to amino acids may be due to mere coincidence, and the birds simply demonstrate the known fact that male broilers grow faster and their finishing weight is higher (Thomas et al., 1977). Another possibility is that the different hormones of females and males have dissimilar effects on the adipose tissue, and the effect of amino acids alters these endocrine changes to a variable degree.

Body fatness can be altered by changing the protein to energy (P/E) ratio. Birds on a marginally deficient diet will fatten as a result of an increased feed intake. Till the limiting factor reaches the requirement, the additional other nutrients will be deposited (Gous, 1986). Poultry can eat more to increase the intake of some limiting nutrients. This happens when the diet contains less protein than would be needed. An increase of the P/E ratio will be accompanied by a decrease in body fat content. Whitehead (1986) suggests that the use of finisher diets of lower energy content is the best way to decrease the body fat percentage. Ambient temperature also plays an important role. Poultry tend to eat less over

15 °C, and in that case unbalanced diets (with an amino acid composition differing from the recommended composition) can cause fat deposition.

Sibbald and Wolynetz (1986) studied the body composition response to different levels of dietary lysine and energy. Their hypothesis was that, for a given feed intake, the amount of energy retained as fat decreases as the lysine supplementation reaches the requirement. They found that the lysine requirement varied depending on the response criteria. It was the lowest for maximal BWG and the highest for maximum protein accretion. The relationship between energy retained as fat and as protein was linear, but the slopes varied with dietary lysine concentrations. Thus, lysine supplementation has an effect on carcass composition independent of energy intake. Sibbald and Wolynetz (1987) also investigated whether the effect of lysine supplementation was modified by added fat. The responses to dietary lysine were found to be independent of the fat content of the diet within the range 20 to 80 g/kg.

D'Mello (1994) stated that feed intake generally increased as the protein level of the broiler diet was lowered. The frequency of feeding should also be taken into consideration. *Ad libitum* feeding causes less fat deposition than a once-a-day feeding regime. It can be concluded that broilers do not eat so strictly for energy as was commonly said of poultry before; rather, they eat for pure nutrients. D'Mello (1994) also mentioned that lysine and isoleucine supply had a striking effect on the fat content of 3-week-old broilers, but the effect could be seen only above a certain amino acid level (threshold).

Holsheimer and Ruesink (1993) wanted to determine the extent to which lysine content and lysine to energy ratio influenced the performance and carcass quality of broilers in different growth periods. The best BWG and feed conversion were achieved during the first 14 days by the group on the highest energy and lysine level. From 3 to 7 weeks of age the highest lysine concentration had a positive effect on the gain. The birds fed a low-energy and normal lysine diet in the first two weeks of life showed the best feed conversion during that period. For the whole growth period the highest BWG was achieved with 1.2 and 1.3% lysine in the diet, and this result was independent of the treatment given in the first two weeks. The finishing diet of 1.3% lysine concentration resulted in the best feed conversion rate. In the first two weeks, the highest carcass accretion was recorded in the group fed the high-energy and lysine-rich diet. For the whole period, the groups receiving 1.2 and 1.3% dietary lysine had the highest protein accretion, regardless of the diet fed in the first two weeks. The above authors clearly showed that the optimal level of lysine and energy varied with the target (maximum BWG, optimum FCR, maximum lean meat production). However, the financial return must also be considered, therefore the market factors should be taken into account when selecting the targets.

At different ambient temperatures, turkeys receiving a lysine-supplemented diet achieved the same BWG while consuming less feed due to a better FCR (Jackson et al., 1988). Velu et al. (1972*b*) described that the fat content of the whole body of chickens increased parallel to the elevation of dietary lysine level if the basal diet was balanced and lysine free. This observation suggests that there is a close correlation between amino acid supply and body composition.

Kwakkel and van Esch (1995) found that the lack of nutrients at a given stage might delay the onset of egg production. Since the first limiting amino acid of poultry growth is lysine, this was used as the major nutrient to restrict. Recently it has been shown that growth in different animal species could be described in terms of distinguishable growth phases or spurts by a multiphase growth model. The control diets contained 0.85 and 0.65% digestible lysine, while the restricted lysine diets 0.4 and 0.3% lysine in starter and grower, respectively. Two other groups were introduced by pair feeding to the restricted lysine group. Body composition at puberty (16.3 weeks for the control) was 3.9% ash, 19.2% crude protein, 61.7% water and 15.2% ether extract. The above authors stated that the amount of fat was not a key factor of pubertal growth. It appears that fat-free components are largely interdependent in terms of their growth. It is concluded that a specific amount of fat-free tissue seems to be critical in determining the moment of development of the reproductive tract. Larbier et al. (1982) studied several amino acids in the body of poultry. They found that arginine and lysine concentration in blood and muscle correlated well with each other but not with the other amino acids studied which were cross-correlated with one another. The highest correlation between blood and muscle concentrations was obtained for lysine of all amino acids; however, individual variation was high. Bedford et al. (1987) investigated hepatic and renal polyamine synthesis. Polyamines (putrescine, spermine and spermidine) play an important role in controlling cell growth and are produced from arginine and methionine. Excess dietary lysine increased renal concentrations of ornithine due to induction of arginase. This accumulation of polyamine precursors resulted in increased concentrations of putrescine, but the BWG and feed intake quadratically decreased.

Growth hormone (GH) gave inverse results in birds as compared to mammals, owing to the different hormonal regulation of lipid and carbohydrate metabolism of avian species. Elevated GH and insulin levels enhance the rate of lipogenesis. Cogburn et al. (1989) treated 3-week-old broiler cockerels with natural and recombinant chicken growth hormone (cGH) for two weeks. The treatments included 100 and 200 μg GH for one kg body weight per day by subcutaneous injection. The chickens responded well. The plasma concentration of GH increased 2- to 6-fold while somatomedin concentration did not change. The BWG and the FCR were the same in the treated animals and the controls, but the treated animals retained more fat (+17%).

The essential status of an amino acid can be judged by determining what percentage of the absorbed amount is used for gluconeogenesis. For growing poultry, the amino acid recommendations of NRC (1994) are 11.0 grams of lysine and 9.0 grams of methionine + cystine in one kg of air-dry feed.

The objective of the present trial was to investigate the body composition of broiler chickens reared on diets of different energy to protein/lysine ratio, measured by direct chemical analysis and computed tomography (CT).

Materials and methods

Animals. Three hundred one-day-old meat-type TETRA-82 hybrid cockerels, purchased from the Agricultural Combine of Bábolna, were used.

Diets. The chickens were fed the following meal-form diets. The unsupplemented basal diet or control (further on: 'B') contained 12.25 MJ ME, 18.7% crude protein, 2.9% ether extract and 9.3 g/kg lysine. The applied supplementations and treatment (group) signs were as follow: 'F' = B + 4% mixed animal fat, 'F-LYS-I' = F + 3 g/kg lysine, 'F-LYS-II' = F + 6 g/kg lysine, 'B-LYS-I' = B + 3 g/kg lysine, and 'B-LYS-II' = B + 6 g/kg lysine.

During the experiment, BWG and feed consumption were measured and FCR was calculated. The growth trial was described in more detail in a previous paper (Fekete et al., 1992). At the end of the growth trial (49 days of age) representative birds from each group were subjected to food hygienic and cooking value tests. The measured parameters included breast muscle weight with bone, leg muscle weight with bone, and amount of abdominal fat. All muscle samples were tested by the cooking-frying test and the consistency test described by Grau (1969).

Ten chickens or representative body weight were selected from each group for CT. The birds were euthanised by carbon dioxide inhalation, deep frozen and individually wrapped in plastic bags. All birds were analysed by CT (for details see Romvári, 1996). After the CT measurements the samples were kept in deep-freezer (at –20 °C) until chemical analyses. Before the final processing the carcasses were thawed overnight at room temperature. The following day the carcasses were manually dissected into pieces of 200 grams (containing both meat and bone as well as skin and feather) and ground twice with a laboratory mill (Type MA-750-C-1™, KERIPAR, Tatabánya) to achieve homogeneity.

Water binding capacity was measured as follows. The total amount of meat was put into a plastic bag. After deep-freezing for 24 h, the outcome water was measured indirectly and expressed in absolute value.

The chemical composition of the samples was determined according to the A.O.A.C. (1975) recommendations. The experimental data were analysed (*t*-comparisons, analysis of variance, calculations of regression and correlation) after Sváb (1981), using the statistical program of the software EXCEL 5.0 (Kovalcsik,

1996). The experiment was approved by the Administrative Advisory Committee on Animal Use and Care of the Hungarian Scientific Chamber.

Results

The live and frozen body weights and the major chemical components of whole chicken bodies on day 49 of life are given in Table 1 and presented in Figs 1–3. The differences between live and frozen weights, which reflect the water-holding capacity, are 193 (12.2%), 162 (9.91%), 178 (10.3%), 151 (9.1%), 125 (7.5%) and 61 (3.6%) grams in the different treatments, respectively (Table 1). The highest frozen body weight was achieved by group B-LYS-II (Figs 1–3).

Table 1

Live and frozen body weight and chemical composition (mean ± SD)

		Live body weight g	Frozen body weight g	Water loss g	Crude protein g/chicken	Ether extract g/chicken	Gross energy MJ/chicken
Control (B)	mean	1583	1390	207	256	191	14
	± SD	170	152	65	38	49	2
	n	10	10				
F (added fat)	mean	1634	1472	162	269	247	16
	± SD	175	47	56	17	33	1
	n	10	7*				
F-LYS-I	mean	1722	1544	178	275	303	18
	± SD	188	183	33	33	43	2
	n	10	10				
F-LYS-II	mean	1667	1516	159	263	280	17
	± SD	182	185	23	44	31	2
	n	10	10				
B-LYS-I	mean	1667	1542	123	287	246	16
	± SD	125	140	16	18	45	2
	n	10	10				
B-LYS-II	mean	1695	1634	61	307	223	16
	± SD	145	125	33	27	35	2
	n	10	10				

*3 samples were not suitable for further analysis (deterioration)

The results of direct chemical analysis for total body and dry matter are shown in Figs 2–3. The body composition of groups F-LYS-I and F-LYS-II was

statistically different ($p < 0.05$) from that of the group receiving the basal diet (B). The difference was 6% and 5% more fat, respectively (Fig. 2). This was parallel to a decrease in the water content of the body, so the difference in fat on dry matter basis was approximately 10% (Fig. 3). The composition of carcasses shows that in treatments designated F, F-LYS-I, F-LYS-II and slightly in B-LYS-I total body water percentage decreased and fat percentage increased as compared to the control (group B; Fig. 2). The body composition of chickens in group B-LYS-II was very similar to the control (B), but both live and frozen final weights were significantly ($p < 0.05$) higher (Table 1). The dry matter content of the different treatments ranged between 36.1 ± 1.3 (B-LYS-I) and 40.8 ± 1.4% (F-LYS-I). Except for these two extreme values the data are not significantly different ($p > 0.05$). Fat supplementation lowered carcass quality by increasing the percentage of body fat. This disadvantage cannot be compensated for by supplementing the diet with 3 or 6 g/kg lysine. The addition of 3 and 6 g/kg lysine to the basal diet improved the performance (BWG) of the chickens proportionally to the lysine supplementation, without altering body composition.

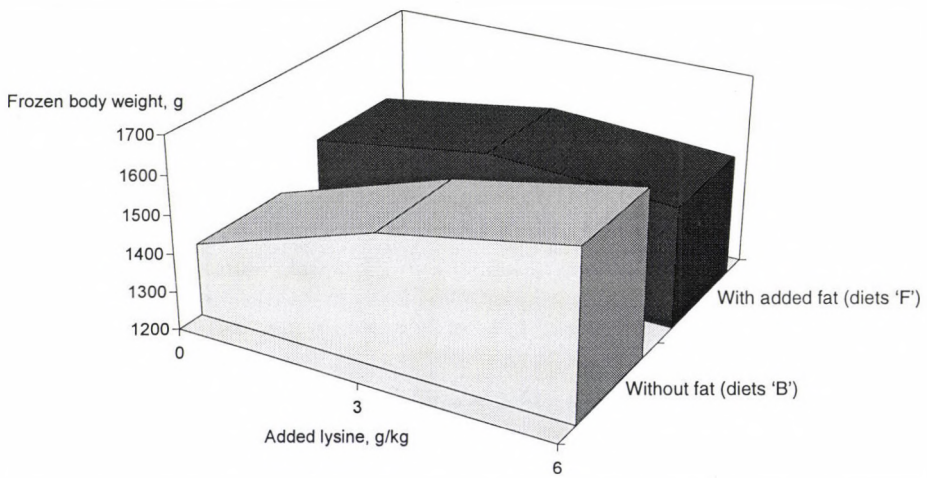

Fig. 1. Frozen body weight as a function of lysine and fat supplementation

The results of linear regression analysis are given in Table 2 and illustrated in Figs 4 and 5. There is a strong negative correlation between the water (x, %) and fat (y_1, %) content of the whole body: $y_1 = 99.471–1.350x$; $n = 57$; $r = –0.877$. That is not the case between whole body water (x, %) and protein (y_2), where the correlation is weak: $y_2 = 3.208 + 0.243x$; $n = 57$; $r = +0.384$. (Minimum-maximum values for water, fat and protein are 59.7–64.6, 13.6–19.7 and 17.3–18.8% of total body, respectively.)

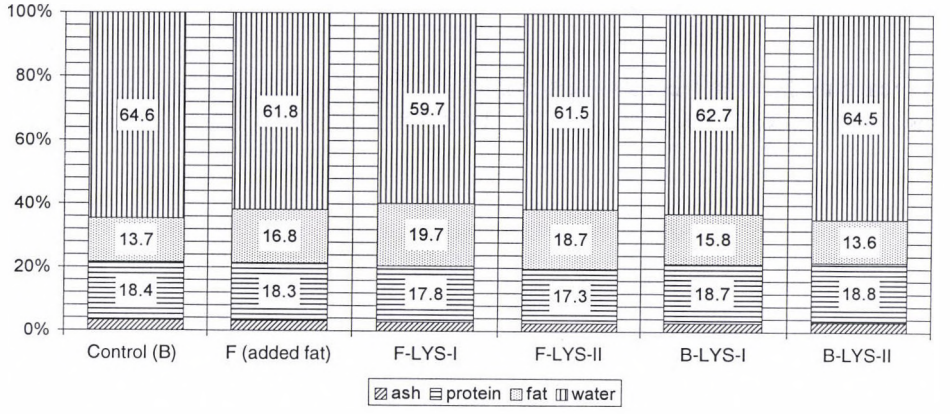

Fig. 2. Chemical composition of total chicken body

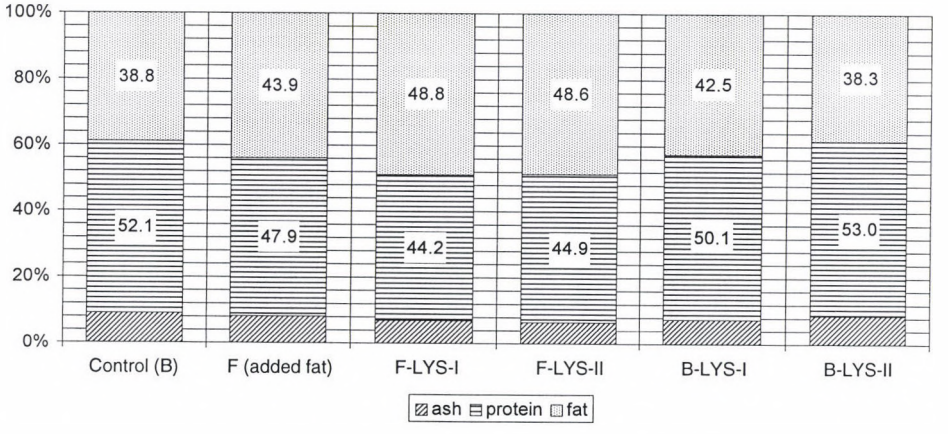

Fig. 3. Chemical composition of the dry matter

Two typical histograms from the results of the CT measurement are shown in Figs 6 (B) and 7 (F-LYS-II). For a better understanding, 1 'H-unit' on the scale corresponds to 10 HU (Hounsfield units): H1 = (–200) to (–191), H2 = (–190) to (–181) ... H40 = (+190) to (+199). A range of H1 to H18 is characteristic of fat, H19 to H21 of water and H22 to H40 of protein/muscle (Horn, 1991). It can be seen on the scans that the detectable differences are tiny.

Table 2

Relationship between water (x, %), fat (y_1, %) and protein (y_2, %) content of whole chicken body on day 49 of life

		Fat/water		Protein/water	
B (control)	n = 10	$y_1 = 118.42–1.658x$	r = –0.747	$y_2 = –20.015+0.509x$	r = 0.426
F (added fat)	n = 7	$y_1 = 55.962–0.642x$	r = –0.354	$y_2 = 37.506–0.314x$	r = –0.277
F-LYS-I	n = 10	$y_1 = 85.381–1.111x$	r = –0.786	$y_2 = 11.042–0.116x$	r = 0.159
F-LYS-II	n = 10	$y_1 = 120.936–1.702x$	r = –0.921	$y_2 = –11.769+0.483x$	r = 0.669
B-LYS-I	n = 10	$y_1 = 76.753–0.982x$	r = –0.661	$y_2 = 24.866–0.103x$	r = –0.130
B-LYS-II	n = 10	$y_1 = 89.304–1.185x$	r = –0.883	$y_2 = –2.645+0.334x$	r = 0.579
	n = 57	$y_1 = 99.471–1.350x$	r = –0.877	$y_2 = 3.208+0.243x$	r = 0.384

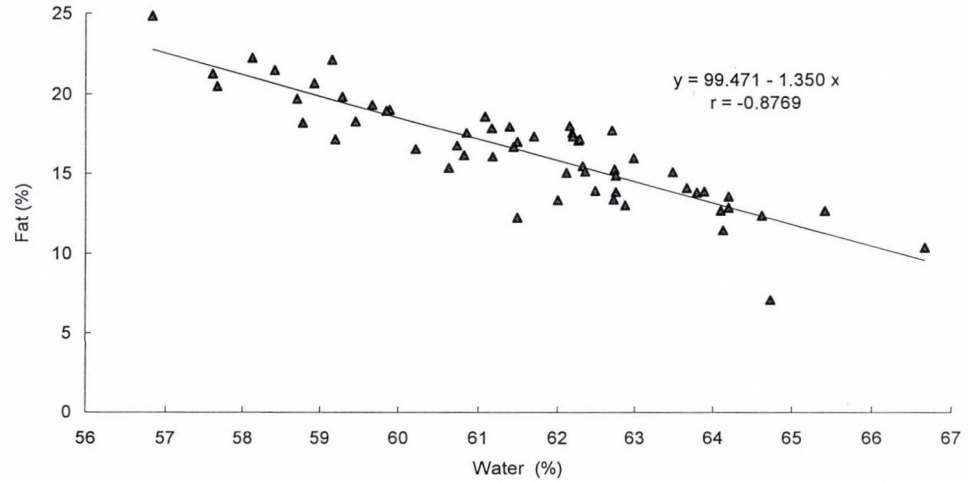

Fig. 4. Relationship between water and fat content of whole chicken body

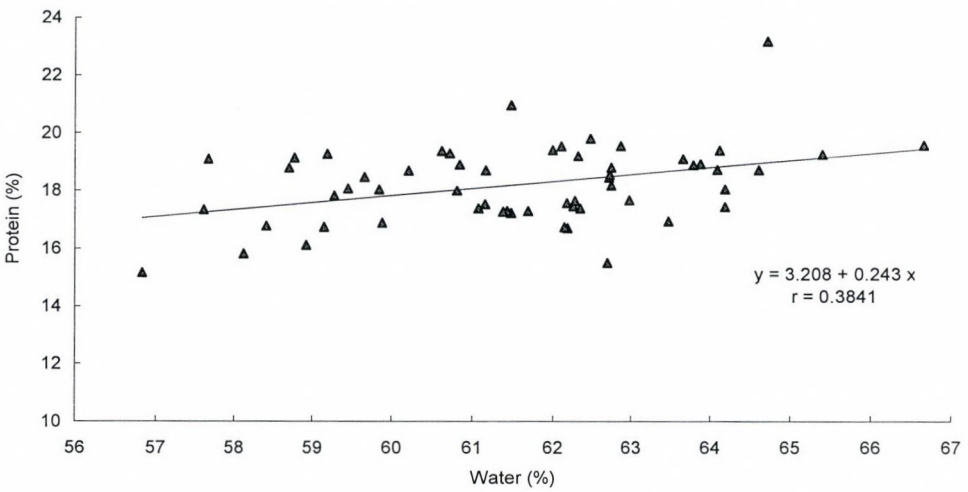

Fig. 5. Relationship between water and protein content of whole chicken body

Discussion

Supplementation of the diet with 6 g/kg lysine did not change the total body composition, but it did influence the final body weight. The latter result was unexpected, because Sibbald and Wolynetz (1986) had found that the lysine concentration of the daily feed influenced the composition of the carcass independently of the energy level. They reported that 9.4 g/kg lysine was sufficient for the maximum growth rate of broilers.

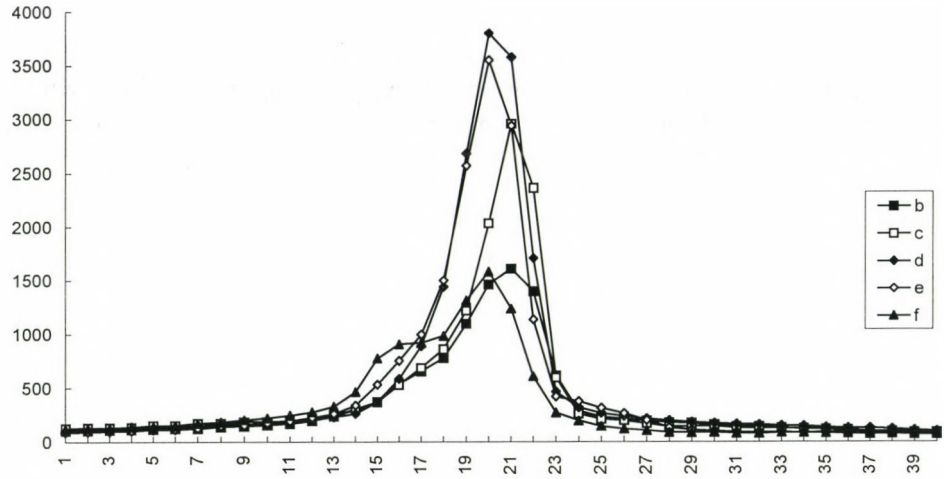

Fig. 6. Histogram of five CT scans (b, c, d, e, f) of frozen bodies of the control group (B)

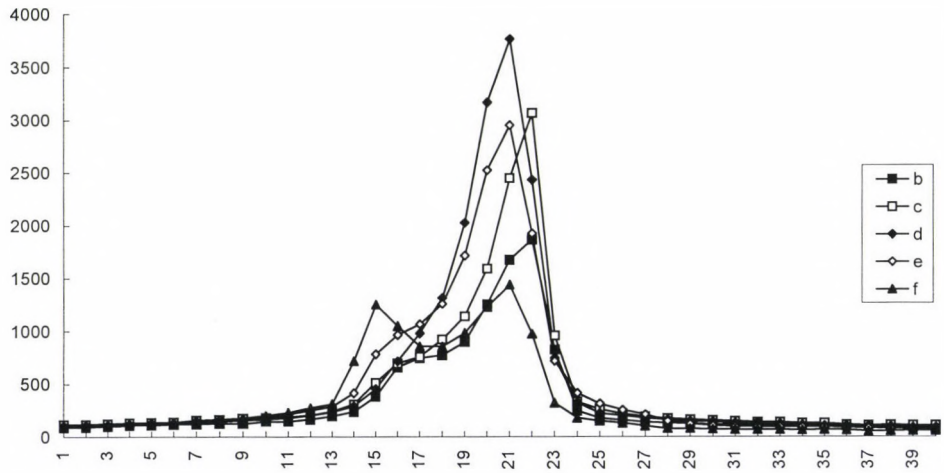

Fig. 7. Histogram of five CT scans (b, c, d, e, f) of frozen bodies of chickens receiving treatment (F-LYS-II)

In a previous paper (Fekete et al., 1992) it was reported that supplementation of the diet with 6 g/kg lysine increased the final frozen weight of broiler chickens from 1390 ± 152 to 1634 ± 125 grams. The calculated lysine concentration of the basal diet was 9.3 g/kg; thus, a lysine concentration of 15.3 g/kg was found to be optimal for rapid lean growth. The body weight gain was proportional in the major chemical compounds (Table 1 and Figs 2 and 3). Due to

fat supplementation the body weight gain increased ($p > 0.05$) parallel to the increase of fat and the decrease of water concentration.

The regressions between the major chemical components of the whole body permit a reliable prediction of fat content from water content, but this statement does not apply to the calculation of protein. The present results are very similar to the fat equation of Velu et al. (1972*a*); however, they found a significant positive correlation between water and protein content as well.

On the basis of the present state of knowledge, the results obtained in the case of fat and 3 g/kg lysine supplementation are difficult to explain. Here the higher body weight ran parallel to a higher fat content. Sibbald and Wolynetz (1986) explain that phenomenon by suggesting that the extra (in their case 3 g/kg) lysine is used as an energy source and its specific action as essential amino acid cannot be realised. If broiler diets are supplemented with energy, they should be supplemented with lysine as well (Vincze, 1993). D'Mello (1994) integrated data of lysine and isoleucine supplementations and concluded that at very low levels of isoleucine or lysine, fat content was relatively low but this increased progressively with graded dietary supplements of amino acids. However, a point was reached for each amino acid when further dietary addition reduced carcass fat concentration. The turning point was at 6–7 g/kg for lysine and at 5 g/kg for isoleucine. In the present study, we had 9.3, 12.3 and 15.3 g/kg lysine levels, and a decline in fat content occurred only at the level of 15.3 g/kg. This may mean that the chicken strain used in this study had a genetically higher amino acid requirement than that of Velu et al. (1972*b*). Among the fat-supplemented groups (F, F-LYS-I, F-LYS-II), a higher lysine addition (6 g/kg) resulted in a lower final body weight than the supplementation of 3 g/kg lysine (live body weight 1634 ± 175, 1722 ± 188 and 1667 ± 182 g, respectively). This can be explained by a relative arginine deficiency caused by lysine–arginine antagonism (Austic, 1986).

The CT measurements proved that body water was transformed into ice during the deep-freezing. The X-ray absorption properties of ice prevented the realistic evaluation of the pictures. The 'ice' peak covers up the reflection of other tissues. As a conclusion, it can be stated that computed tomography is not appropriate for the measurement of deep-frozen material.

The difference between frozen body weight and final live body weight shows that the water-holding capacity of muscle was increased by the lysine supplementation, and it was the best in groups with a normal energy and high lysine supplementation (B-LYS-II). This must also be reflected in meat quality. This finding is consistent with the statement that the different criteria have dissimilar recommendations in terms of amino acid requirements (Baker, 1989).

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STUDIES ON THE OCCURRENCE OF ACTINOSPOREAN STAGES OF FISH MYXOSPOREANS IN A FISH FARM OF HUNGARY, WITH THE DESCRIPTION OF TRIACTINOMYXON, RAABEIA, AURANTIACTINOMYXON AND NEOACTINOMYXON TYPES

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Actinosporean infection of the oligochaete fauna living in the mud and on the vegetation of fish ponds used for rearing common carp in polyculture was studied during a one-year survey at a fish farm in Hungary, located south of Budapest. Twenty-eight actinospore types were isolated from the oligochaetes *Tubifex tubifex, Branchiura sowerbyi, Limnodrilus hoffmeisteri, Nais elinguis,* and *Stylaria lacustris* collected during the survey, which could be classified into the triactinomyxon, raabeia, aurantiactinomyxon and neoactinomyxon groups. Drawings depicting individual actinospore types are presented on plates and their characteristic dimensions have been summarised in tables. The prevalence and seasonality of actinosporean infections observed in the various oligochaete species have been illustrated graphically. Infection by actinospores showed a pronounced seasonality. In the spring, summer and autumn the prevalence of raabeia infection in *Branchiura* exceeded 90%, while in the winter it dropped to 42%. A similar phenomenon could be observed for aurantiactinomyxon infection, while neoactinomyxon infection reached its peak in the autumn. In *Tubifex, Limnodrilus, Nais* and *Stylaria* species the peak of actinosporean infection occurred, with minor differences, in the spring and summer. Actinosporean infection in the individual Oligochaeta species showed much higher prevalence values than had been reported in the literature, which can be explained by the novelty of the examination technique used. It cannot be decided with absolute certainty which myxosporean developmental stage the different actinospore types described during this survey represent of the species of Myxosporea parasitic in the given ponds. This would require an experimental study for which the data presented here may serve as a basis.

Key words: Actinospore, myxosporeans, Myxozoa, survey, triactinomyxon, raabeia, aurantiactinomyxon, neoactinomyxon

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The first report on actinosporeans was published by Stolc (1899), who described these organisms found in Bohemia as parasites related to myxosporeans. Although nearly one hundred years have elapsed since their first description, until quite recently only few researchers had studied actinosporeans. Of them, Ikeda (1912) and Mackinnon and Adam (1924) detected tetractinomyxon and triactinomyxon forms in England, while in Poland Janiszewska (1955, 1957) performed detailed studies on the morphology, ecology and systematics of actinosporeans. The ultrastructure and biology of these parasites have been studied most intensively by Marques (1984), Lom and Dykova (1997), and Lom et al. (1997*a*). Research on actinosporeans gathered momentum after Wolf and Markiw (1984) had demonstrated that actinosporeans did not constitute an independent taxonomic unit but corresponded to fish-parasitic myxosporean stages developing in oligochaetes. After Wolf and Markiw (1984), other researchers also produced experimental evidence that in the developmental cycle of a given species of Myxosporea the intra-piscine myxosporean and the intra-oligochaete actinosporean stages alternated (El-Matbouli and Hoffmann, 1989; Ruidisch et al., 1991; El-Matbouli et al., 1992; Grossheider and Körting, 1992; El-Matbouli and Hoffmann, 1993; Kent et al., 1993; Yokoyama et al., 1993*a*; El-Matbouli et al., 1995; Yokoyama et al., 1995; Uspenskaya, 1995; Trouillier et al., 1996; Yokoyama, 1997; El-Mansy and Molnár, 1997; El-Mansy and Molnár, in press). Relying on these studies, Kent et al. (1995) proposed that the class Actinosporea should be merged into the class Myxosporea as a synonym of the latter, and that the names of actinosporean genera should in the future be used only for typing actinospores developing in oligochaetes. The major taxonomic changes of recent years are reflected in the works of Smothers et al. (1994), Kent et al. (1995), Siddal et al. (1995), and Schlegel et al. (1996), who reassigned phylum Myxozoa, earlier regarded by many authors as protozoa, from protozoan to metazoan parasites.

The actinosporean infection of oligochaetes in fish farms and natural waters in relation to the developmental cycle of myxosporeans has so far been studied among others by Hamilton and Canning (1987), Burtle et al. (1991), Székely (1991), Styer et al. (1992), Pote and Waterstrat (1993), Koller (1994), Pallós (1995), and McGeorge et al. (1997).

The objective of the present study was to gain a better understanding of the actinosporean stages of species of Myxosporea occurring in Hungary. A one-year survey was carried out to determine the occurrence of actinosporean stages in water samples taken from the ponds of a fish farm as well as in oligochaetes collected from the ponds.

Material and methods

The survey was carried out at a fish farm situated south of Budapest, between March 1996 and March 1997. From three selected ponds of the farm and from their drain channels oligochaete-containing water-weed and mud samples as well as water samples were taken at biweekly intervals. In the period of study, and also previously, polycultural fish breeding was conducted in the given ponds; however, fish species other than those reared in the ponds could also enter the farm with the inflow water. The water-weed, mud and water samples collected from the ponds were transported to the laboratory for the isolation of oligochaetes and then actinospores.

Preparation of oligochaetes for examination for actinospores

Oligochaetes of small size (3–30 mm) were isolated from the plants and from particles floating in the water under stereomicroscope in Petri dishes, identified to the genus or species level, then placed into small (0.5–5 l) aerated aquaria, using a separate aquarium for each species. Oligochaetes of large body size (40–150 mm) were washed out from the mud, assorted under stereomicroscope, then placed into small aerated aquaria in groups separated by species. Before filling the aquaria with tap-water, mud sterilised by boiling was layered onto the bottom of the aquaria to give a 2–3 cm thick layer. The oligochaetes placed into the aquaria were fed a few granules of fish food twice a week. Chicken faeces was added to the water to increase the organic matter content of the mud.

In the course of the one-year survey the following oligochaete species were examined for the occurrence of actinospores: *Branchiura sowerbyi* (Beddard), *Tubifex tubifex* (Müller), *Limnodrilus hoffmeisteri* (Claparede), *Nais elinguis* (Müller) and *Sylaria lacustris* (L.) [identified according to Brinkhurst (1963)].

Examination of oligochaetes for actinosporean infection. Morphology and differentiation of the actinospore types

After the identification of oligochaetes to the species or genus level, they were examined for the presence of actinospores by the cell-well plate method of Yokoyama et al. (1991). A 24-well plate was used, with wells having a volume of 2 ml and containing tap-water. The worms were placed one by one into the wells which then were covered with a plastic sheet to prevent worms from climbing from one well into another. The plates were kept in thermostat at a temperature of 15 °C and examined under compound microscope for the possible presence of actinospores released from the worms and floating in the water. Any floating actinospores found were examined under an Olympus research microscope at a higher magnification and then recorded on videotape with the help

of a microscope video attachment. Subsequently still images of the spores were taken from the video recordings with the help of a video image program (Imago®). In many cases, simultaneously with the video recordings, photographs were also taken of the actinospores with the help of a conventional photographic attachment. Subsequently drawings were made of the actinospores and their measurements were taken.

Some of the oligochaetes were lifted out of the wells, carefully placed onto slides and examined under a coverslip, in live state, for the presence of actinosporean developmental stages by microscopy. When such stages were found, the live material was first recorded on videotape. Subsequently the infected worms were fixed in Bouin's solution for 4 h, embedded in paraffin, and 3–4 μm thick sections were prepared from them. The sections were stained with haematoxylin and eosin, then examined under an Olympus research microscope to determine the location of spore development.

If the oligochaetes were negative on first examination, the plates containing them were placed back into the thermostat and then re-examined every two days until they released actinospores. Those worms were considered negative which did not release spores from their body even after 3 months. The majority of large oligochaetes (*Branchiura, Tubifex, Limnodrilus*) survived in the thermostat for 3–4 months even without feeding; in their case only water had to be replenished at each examination. The prevalence of infection of the worms was recorded at each examination and the data were summarised by season.

The characteristic dimensions of actinospores recorded on videotape (polar capsules, spore body, style, caudal processes, whole length) were measured with the help of the Imago program. In addition, these measurements were also taken subsequently with the help of a scale recorded on the videotape together with the actinospores separately for each magnification (Székely, 1997). The actinospore types were compared to the actinospore forms reported in the literature earlier (Janiszewska, 1955, 1957; Marques, 1984; Yokoyama et al. 1993*a,b*, 1995). Measurements were always taken on the basis of the average values measured for 10–50 mature spores. The measurements of actinospore types have been summarized in a table according to the guidance given by El-Matbouli (1988) and Lom et al. (1997*b*).

Filtration of pond-water

Due to technical difficulties (high floating alga count, etc.), direct filtration of water from the ponds was attempted in a few cases only. In the majority of cases, especially when examining oligochaetes of small body size, mud and water-weed samples were brought to the laboratory, together with particles floating in the water which often contained specimens of *Nais elinguis* and *Sty-*

laria lacustris. In the laboratory these samples were placed into aquaria. On every second day one litre of water was taken from each of these aquaria and filtered through a mesh of 10 μm pore size for examination for actinospores. Using a small volume of water, the filtrate was then washed off the filtering material onto a slide, and examined for the presence of actinospores at low magnification under a microscope.

Myxosporeans occurring in the fish ponds surveyed

Since the fish farm studied is conducting primarily common carp breeding in polyculture, the commonest farmed fish species are the common carp and the Japanese coloured carp (*Cyprinus carpio*), the silver carp (*Hypophthalmichthys molitrix*), the bighead (*Aristichthys nobilis*), the grasscarp (*Ctenopharyngodon idella*), the goldfish (*Carassius auratus auratus*), and, as complementary predators, first of all the pikeperch (*Stizostedion lucioperca*), the pike (*Esox lucius*), the European catfish (*Silurus glanis*), and the sterlet (*Acipenser ruthenus*). Naturally, non-farmed fish species other than those listed above also gain entry to the fish ponds with the inflow waters in low numbers.

The fish species bred at the farm have been subjected to regular health checks for several years. These checks have detected the following main species of Myxosporea in the fish farm concerned (unpublished):

Common carp (*Cyprinus carpio*): *Myxobolus cyprini, M. dispar, M. basilamellaris, M. encephalicus, Thelohanellus nikolskii, T. hovorkai, Hoferellus cyprini, Sphaerospora renicola, S. molnari.*

Goldfish (*Carassius auratus*): *Hoferellus carassii, Sphaerospora renicola.*

Silver carp (*Hypophthalmichthys molitrix*): *Myxobolus pavlovskii, M. drjagini, Myxobilatus* sp., *Sphaerospora* sp., *Chloromyxum* sp.

Bighead (*Aristichthys nobilis*): *M. pavlovskii, Myxobilatus* sp., *Sphaerospora* sp., *Chloromyxum* sp.

Grasscarp (*Ctenopharyngodon idella*): *Chloromyxum* sp., *Myxidium* sp.

Pike (*Esox lucius*): *Myxobolus anurus, Henneguya psorospermica, Myxidium lieberkuehni.*

Sterlet (*Acipenser ruthenus*): *Sphaerospora colomani, Chloromyxum inexpectatum*

Pikeperch (*Stizostedion lucioperca*): no data are available.

Results

Occurrence of actinospore types in oligochaetes collected from the fish ponds

A total of 28 actinospore types (Figs 1–4 and 12–13) were isolated from the 889 oligochaete specimens collected from the ponds, representing 5 species (*Branchiura sowerbyi*, *Tubifex tubifex*, *Limnodrilus hoffmeisteri*, *Nais elinguis* and *Stylaria lacustris*). The actinospores can be classified into four main groups (triactinomyxon, raabeia, aurantiactinomyxon and neoactinomyxon). The specimens of *Branchiura sowerbyi* exhibited the most prevalent infection, and were in many cases infected by several actinospore types at the same time. Of the 253 *Branchiura* specimens examined, 215 (85%) were infected by raabeia, 149 (59%) by aurantiactinomyxon, and 50 (20%) by neoactinomyxon type actinospores (Fig. 5). Triactinomyxon type infection was not detected in *Branchiura*. Of the 338 *Tubifex* specimens examined, 108 (32%) were infected by triactinomyxon, 4 (1.2%) by raabeia, and 3 (0.9%) by aurantiactinomyxon (Fig. 6). Of the 193 *Limnodrilus* specimens, 65 (34%) were infected by aurantiactinomyxon and 49 (25%) by triactinomyxon (Fig. 7). Neither raabeia nor neoactinomyxon type infection was found in the *Limnodrilus* specimens. Four out of the 31 *Nais elinguis* specimens examined (13%) were infected exclusively by triactinomyxon (Fig. 7), while 3 out of the 74 *Stylaria* specimens examined (4%) was also found to have exclusively triactinomyxon infection (Fig. 7).

Infection by actinospores exhibited a pronounced seasonality. In the spring, summer and autumn the prevalence of raabeia infection in *Branchiura* exceeded 90% (Fig. 8), while in the winter it dropped to 42%. A similar phenomenon was observed for aurantiactinomyxon infection, with the difference that in this case the spring and summer peaks were around 80% and the prevalence of infection dropped to around 40% by the autumn and to a level as low as 14% by the winter. It was interesting to note that neoactinomyxon infection was at a low level in the spring and summer, reaching a high (over 90%) peak in the autumn, followed by a marked decrease during the winter. For the species *Tubifex, Limnodrilus, Nais* and *Stylaria* the highest prevalence of actinospore infection occurred, with minor differences, in the spring and summer (Figs 9–11).

2. Filtration of pond-water

After filtration of water samples from the ponds triactinomyxon, raabeia, aurantiactinomyxon and neoactinomyxon type actinospores were detected (Figs 1–4, Tables 1–4), which were often identical with the actinospore types directly obtained from the worms.

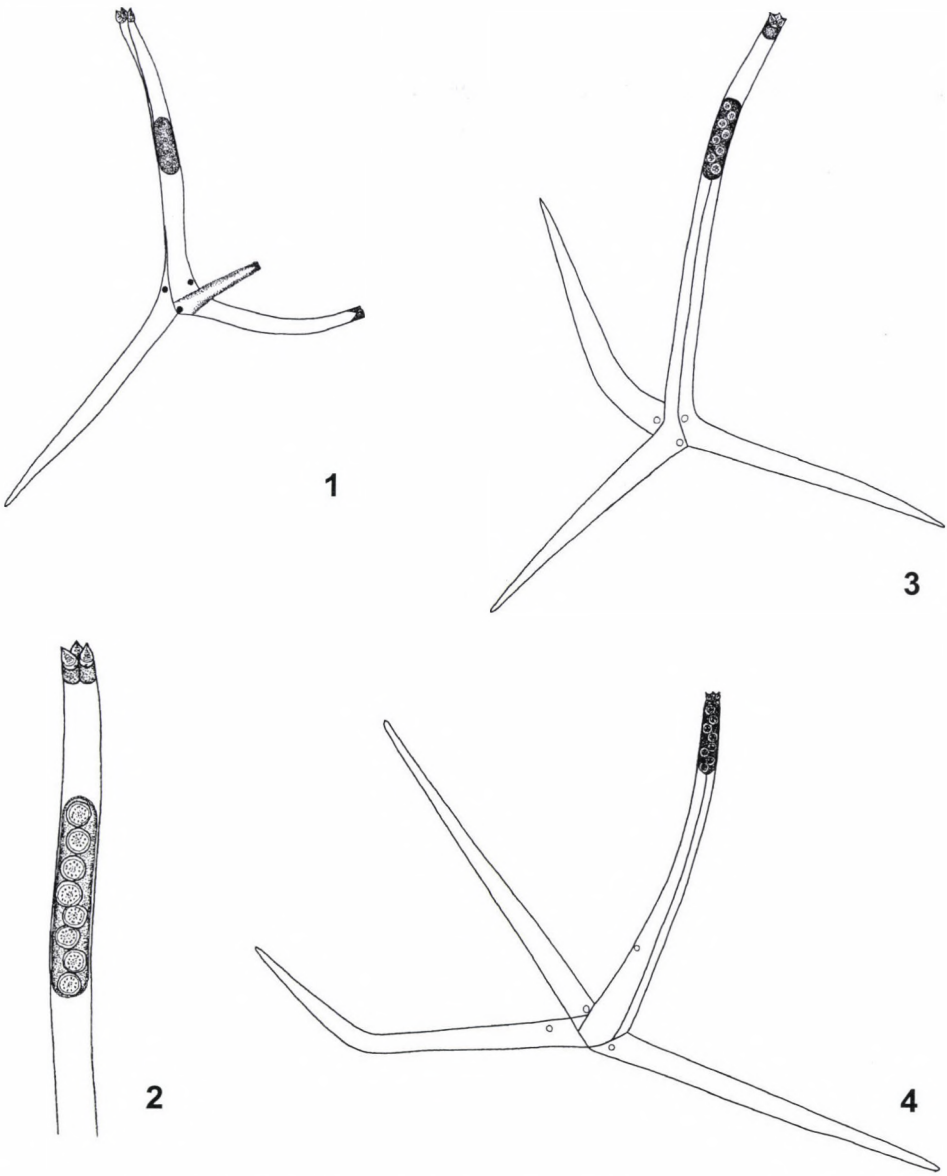


Fig. 1. Line drawings of triactinomyxon types collected from a Hungarian fish farm

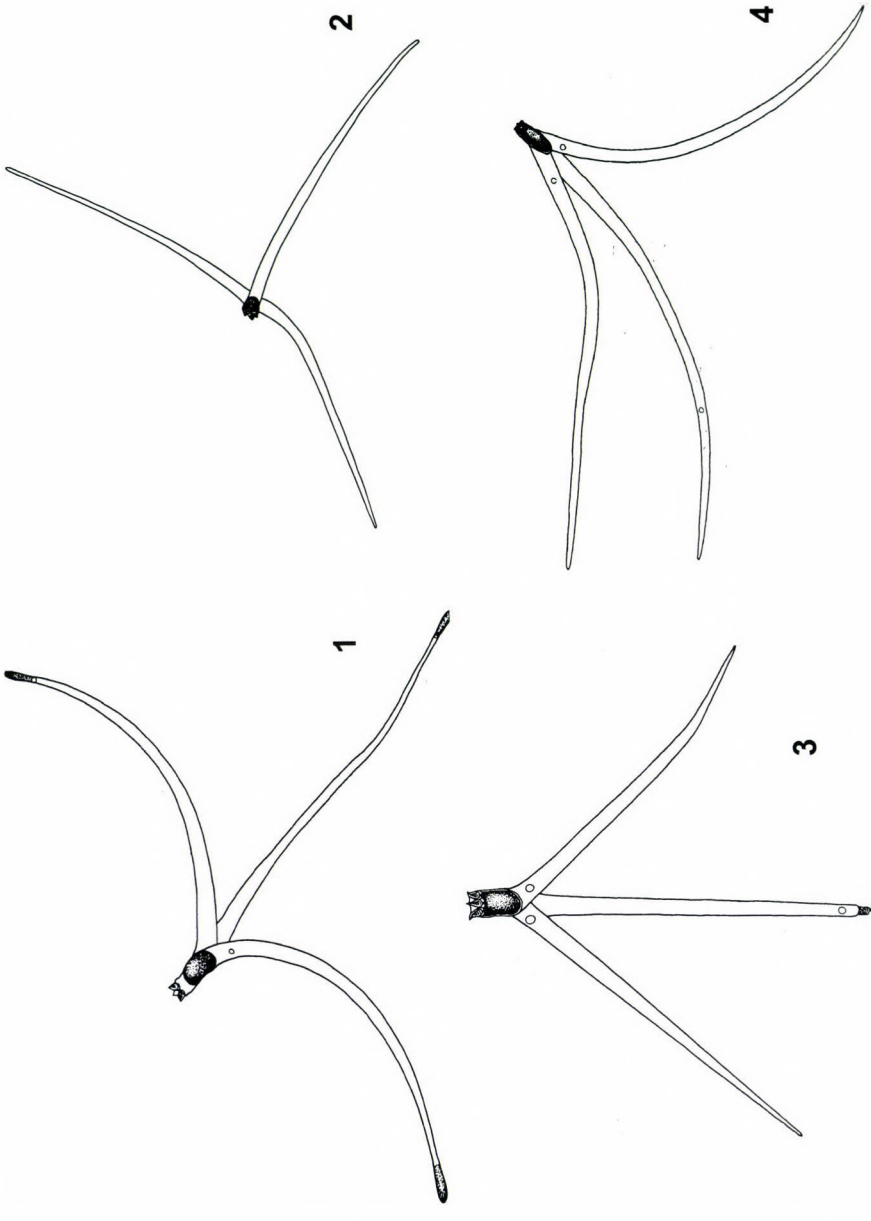


Fig. 2. Line drawings of raabeia types collected from a Hungarian fish farm

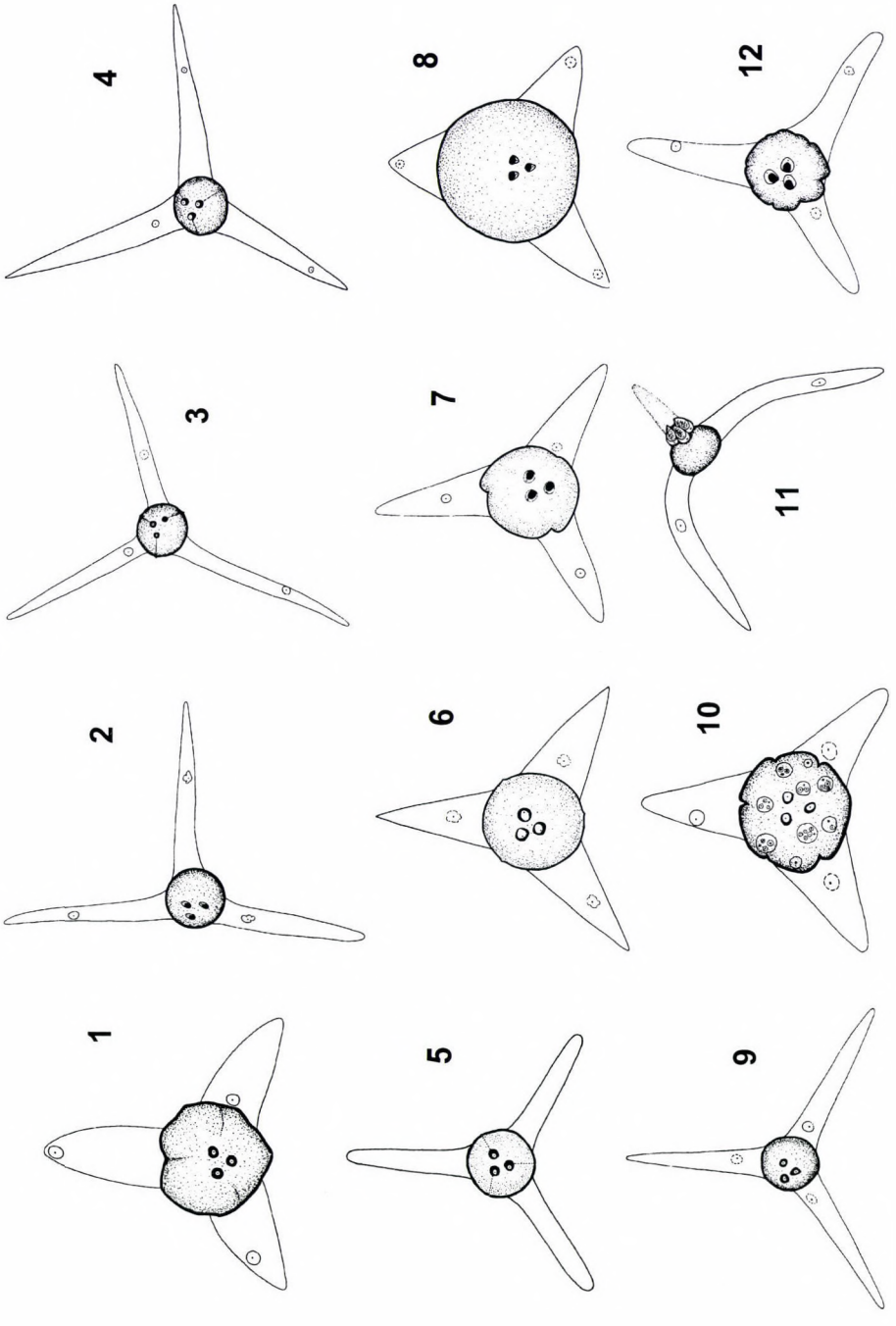


Fig. 3. Line drawings of aurantiactinomyxon types collected from a Hungarian fish farm

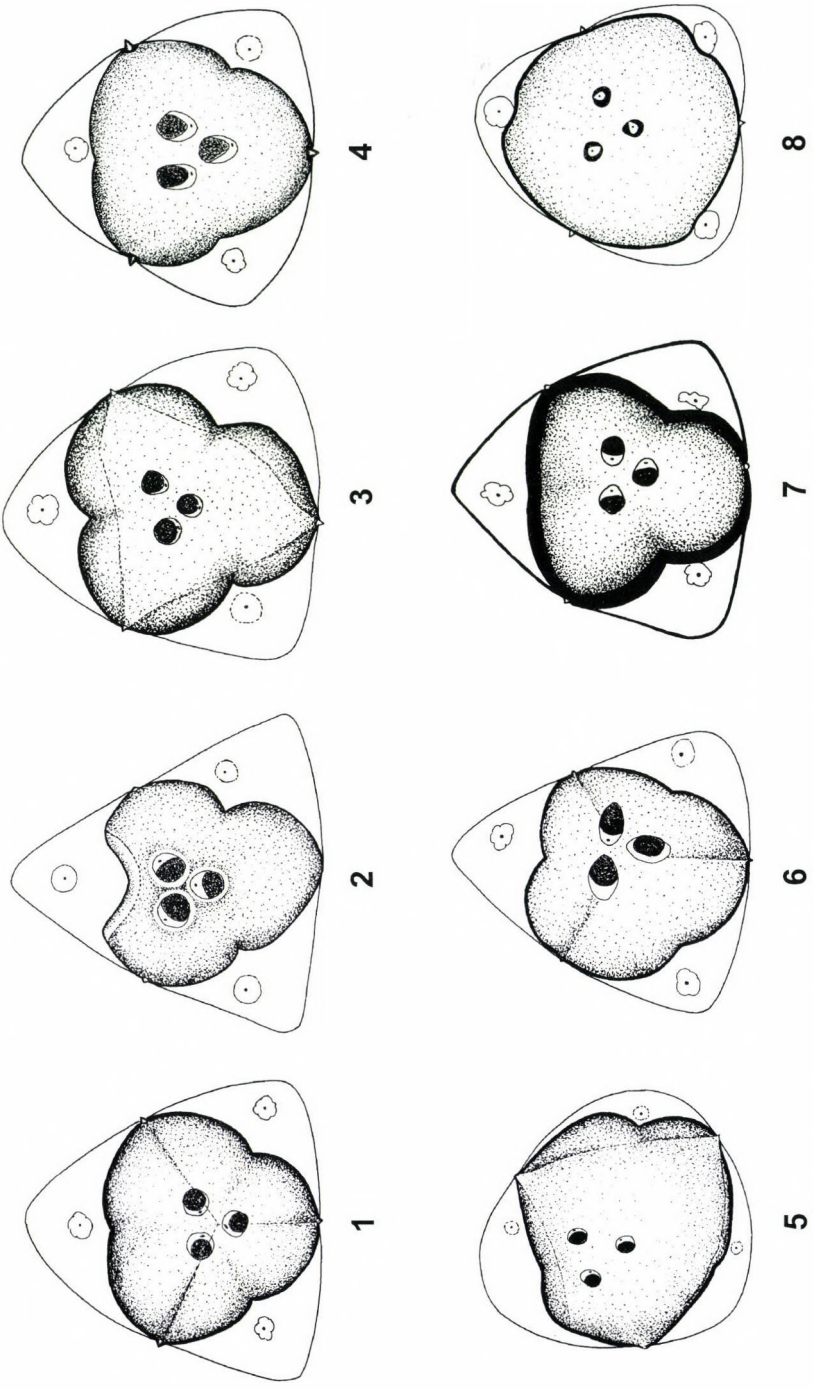
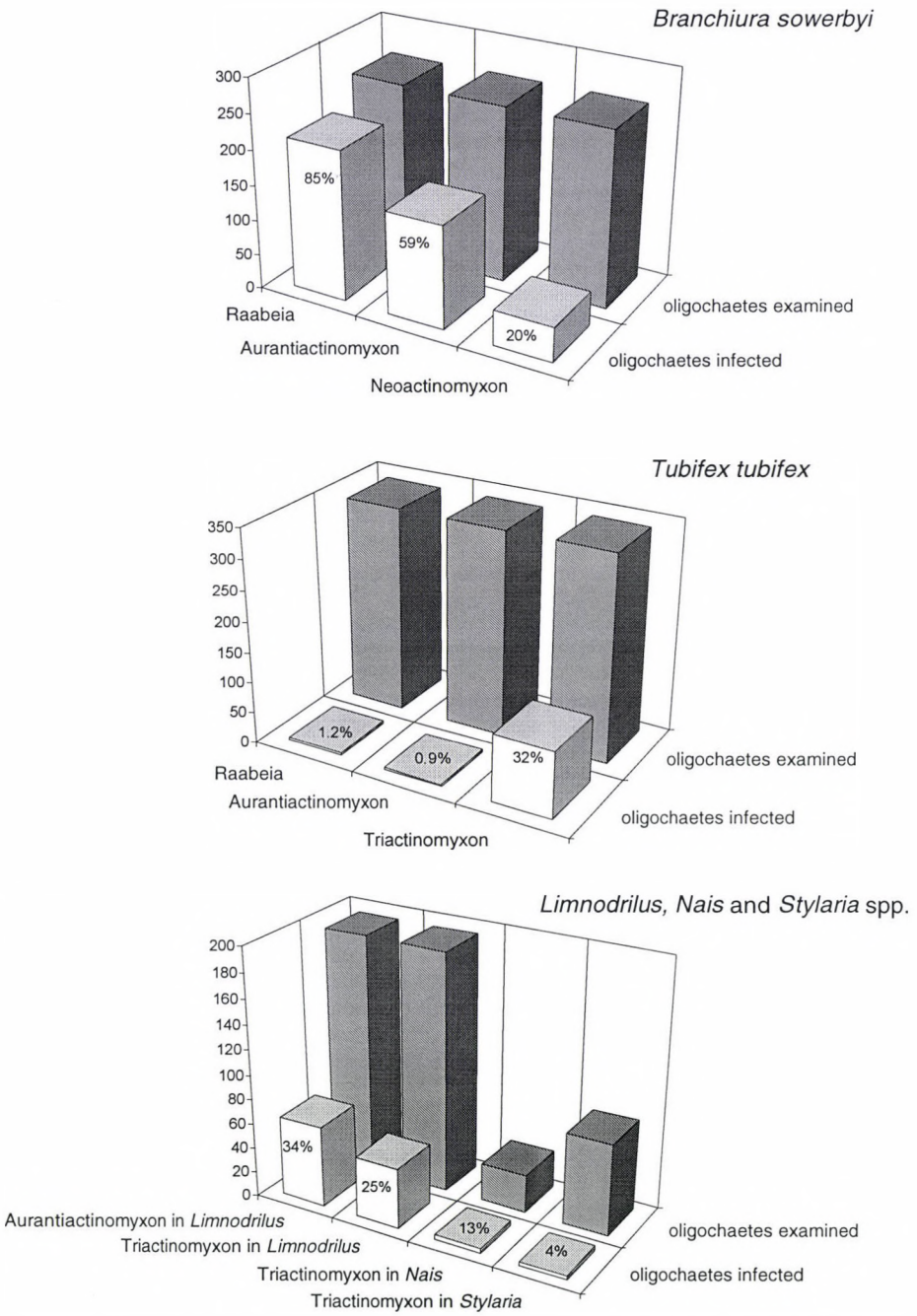


Fig. 4. Line drawings of neoactinomyxon types collected from a Hungarian fish farm



Figs 5–7. All-year prevalence of actinosporean infection of oligochaetes in a Hungarian fish farm from March 1996 to March 1997

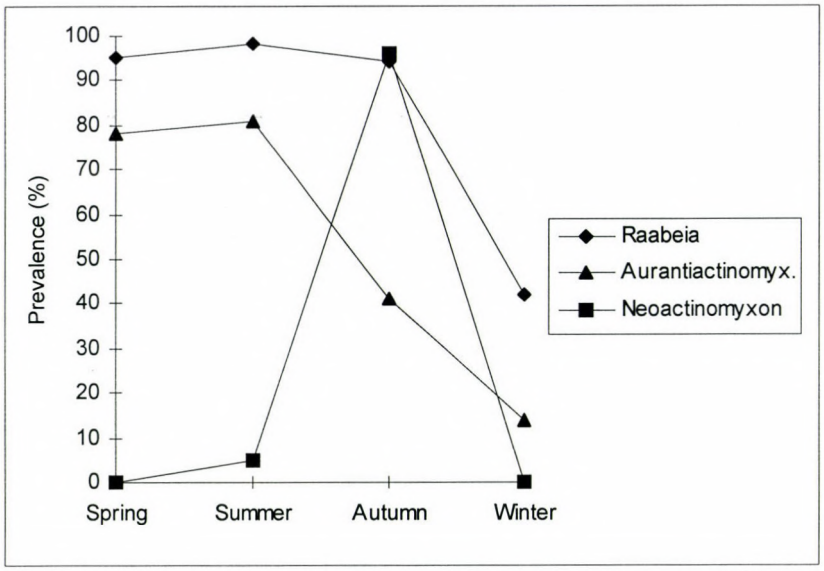


Fig. 8. Branchiura sowerbyi

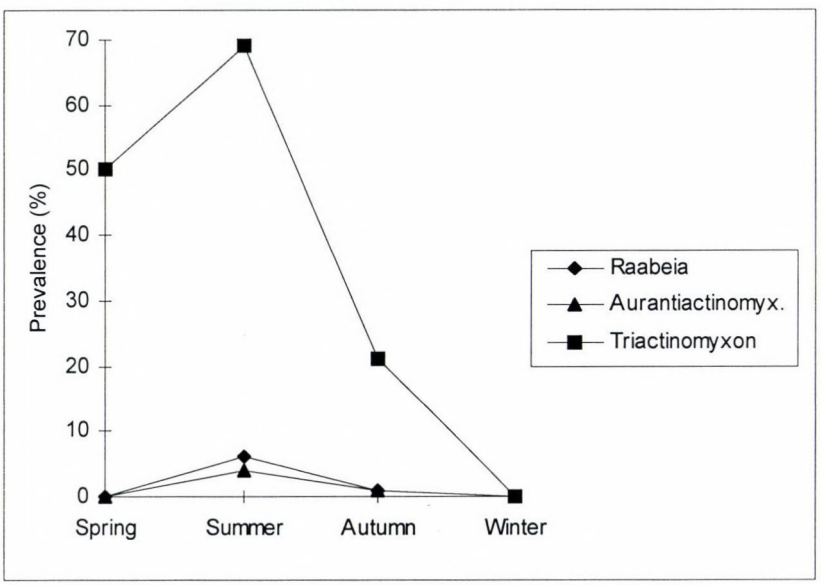


Fig. 9. Tubifex tubifex

Seasonality of actinosporean infection in oligochaetes in a Hungarian fish farm from March 1996 to March 1997

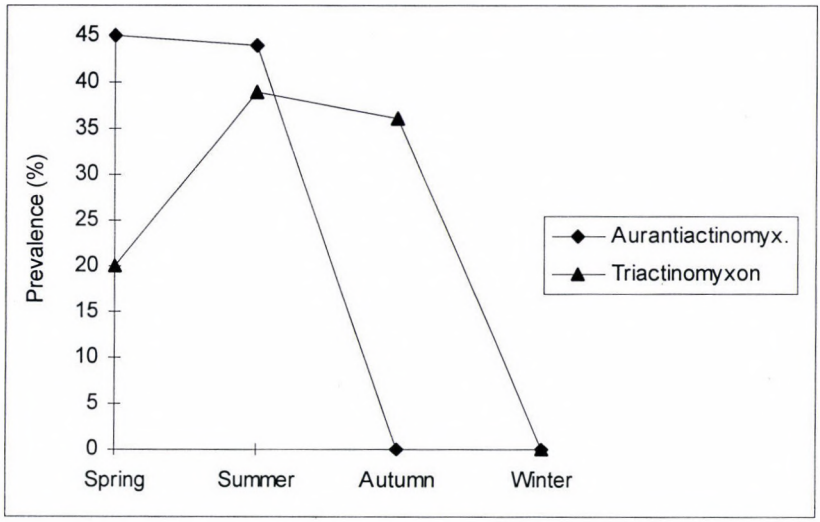


Fig. 10. Limnodrilus hoffmeisteri

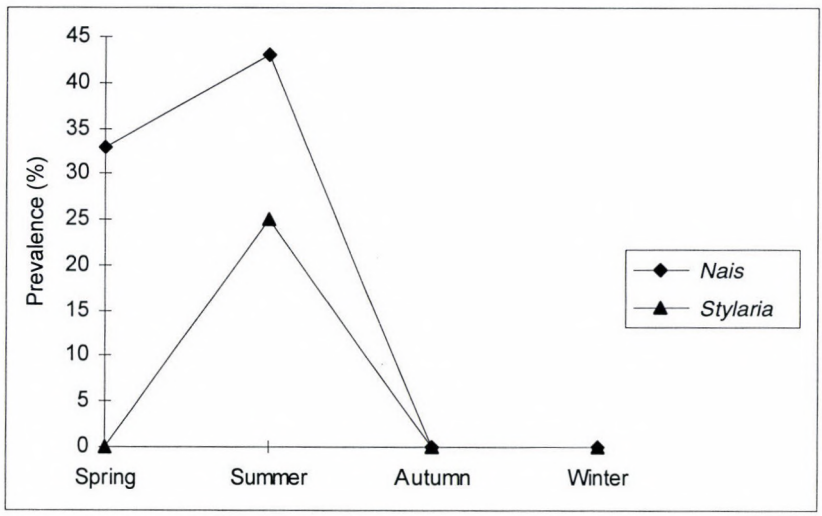


Fig. 11. Triactinomyxon infection in *Nais elinguis* and *Stylaria lacustris*

Seasonality of actinosporean infection in oligochaetes in a Hungarian fish farm from March 1996 to March 1997

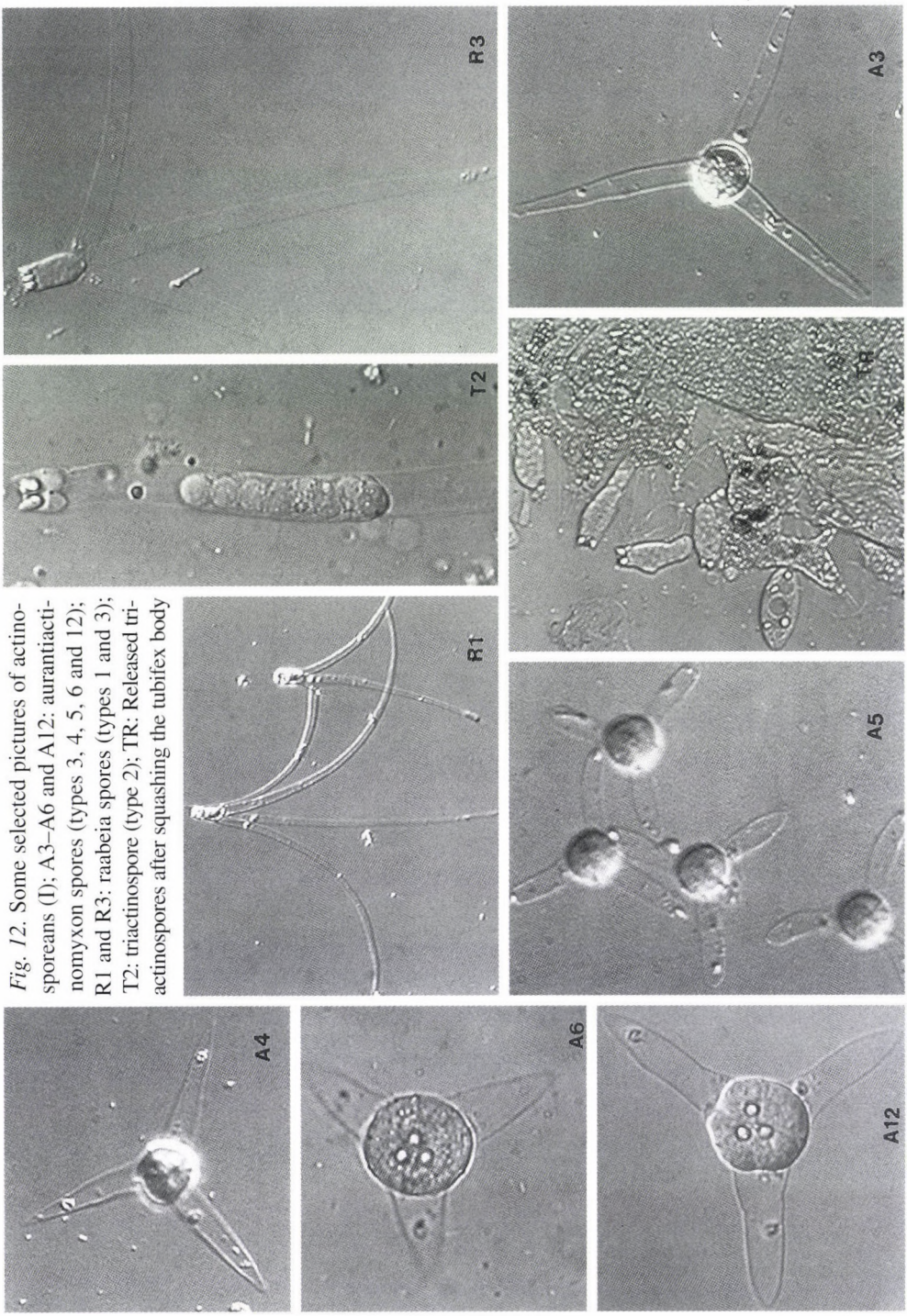


Fig. 12. Some selected pictures of actinosporeans (I); A3–A6 and A12: aurantiactinomyxon spores (types 3, 4, 5, 6 and 12); R1 and R3: raabeia spores (types 1 and 3); T2: triactinospore (type 2); TR: Released triactinospores after squashing the tubifex body

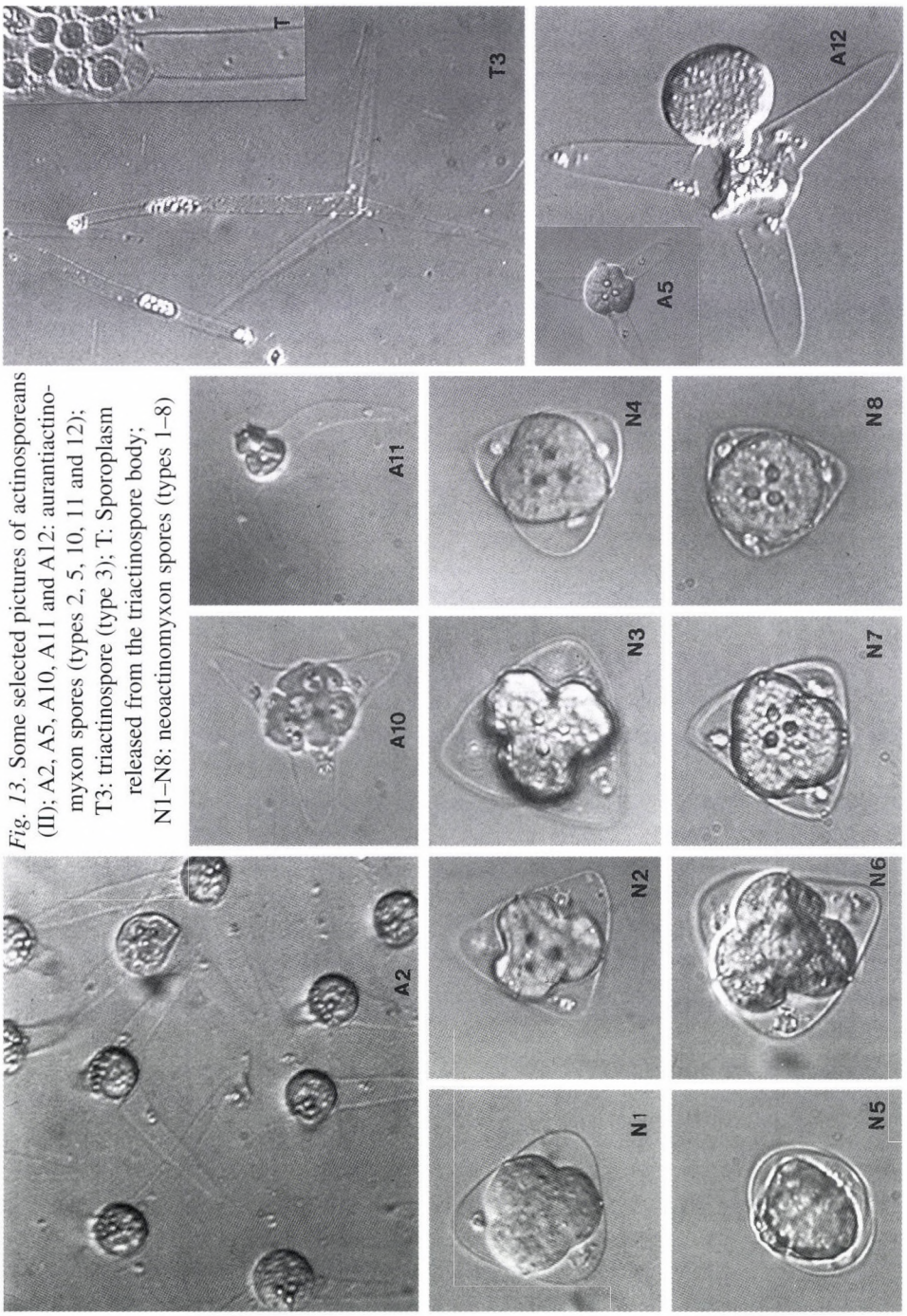


Fig. 13. Some selected pictures of actinosporeans (II); A2, A5, A10, A11 and A12: aurantiactinomyxon spores (types 2, 5, 10, 11 and 12); T3: triactinospore (type 3); T: Sporoplasm released from the triactinospore body; N1–N8: neoactinomyxon spores (types 1–8)

Table 1

Average dimensions of triactinomyxon types found during the survey (in μm)

Triactino-myxon type No.	Origin of spores	Polar capsule dimensions	Spore body dimensions	Secondary cell dimensions	No. of secondary cells	Style length	Style width at start/at end	Caudal process dimensions	Whole length
1	water, *Stylaria, Tubifex*	7.0×3.5	36.6×10.6	n. d.	≈ 27	102	9.4/16.5	128×10.6	236.3
2	water	11.8×5.9	101.2×14.1	11.8×10.6	n. d.	n. d.	n. d.	n. d.	n. d.
3	*Nais, Tubifex*	5.9×4.5	47.1×10.6	6.0×4.7	8	150	15.3/10.6	127.5×14.5	399.4
4	water, *Limnodrilus*	4.7×2.4	41.2×8.8	5.9×4.7	8	137.7	9.4/20.0	173.4/14.3	352.3

n. d. = not determined

Table 2

Average dimensions of raabeia types found during the survey (in μm)

Raabeia type No.	Origin of spores	Polar capsule dimensions	Sporoplasm dimensions	Spore body dimensions	Caudal process dimensions	Whole length (from polar capsules to the end of caudal process)
1	water, *Branchiura, Tubifex*	5.9 × 3.5	20 × 11.8	25.9 × 11.8	294 × 9	320
2	water, *Branchiura*	5.9 × 4.7	8.2 × 12.4	14.1 × 12.4	202.8 × 8.2	216.9
3	*Tubifex*	7.5 × 5.9	18.8 × 12.9	28.2 × 14.1	183.6 × 10.6	211.8
4	water	5.7 × 4.0	16.0 × 6.8	21.7 × 7.7	209.4 × 6.6	231.1

Table 3

Average dimensions of aurantiactinomyxon types found during the survey (in μm)

Aurantiactino-myxon type No.	Origin of spores	Caudal process length and width near the sporoplasm	Polar capsule dimensions	Spore cavity diameter	Largest span
1	*Tubifex tubifex*	17.5 × 9.9	2 × 2	18.3	45.4
2	water,*Branchiura sowerbyi*	65.7 × 10.5	4 × 1.7	22.8	142.5
3	water, *Branchiura sowerbyi*	70.3 × 8.0	2.9 × 2.9	22.8	149.3
4	water, *Branchiura sowerbyi*	55.7 × 11.2	2.9 × 2.9	19.4	122
5	water, *Branchiura sowerbyi*	17.2 × 3.9	1.4 × 1.4	9.9	39.5
6	*Limnodrilus* sp.	24.2 × 11.2	2.8 × 2.8	19.7	55.6
7	water	24.4 × 9.5	2.8 × 2.5	18.9	58.4
8	*Limnodrilus* sp.	12.2 × 9.0	1.4 × 1.4	22.6	39.8
9	water, *Branchiura sowerbyi*	51.3 × 9.5	2.3 × 2.3	18.8	103.2
10	water, *Branchiura sowerbyi*	16.7 × 8.8	1.7 × 1.7	15.5	39.5
11	water	31.9 × 3.7	3.4 × 2.0	8.5	46.5
12	water, *Branchiura sowerbyi*	26.5 × 8.7	2.8 × 3.1	12.1	59.2

Table 4

Average dimensions of neoactinomyxon types found during the survey (in μm)

Neoactinomyxon type No.	Origin of spores	Polar capsule dimensions (length × width)	Spore body width (angle to base)	Caudal process dimensions (length × width)	Span between two caudal processes
1	*Branchiura sowerbyi*	2.5 × 2.8	21.2	8.5 × 16.4	29.3
2	*Branchiura sowerbyi*	3.1 × 2.8	18.3	10.8 × 14.4	31
3	*Branchiura sowerbyi*	2.8 × 2.3	22	8.5 × 16	30.2
4	*Branchiura sowerbyi*	3.7 × 2.8	22.3	7 × 16	29
5	*Branchiura sowerbyi*	1.7 × 1.7	21.2	4.4 × 13.6	23.1
6	*Branchiura sowerbyi*	4.2 × 2.8	20.3	7.8 × 12.7	29.3
7	*Branchiura sowerbyi*	2.8 × 3.1	22.6	8.5 × 16.4	31.5
8	*Branchiura sowerbyi*	2 × 2	22.8	4.2 × 11.3	26.8

Light microscopy

Actinosporeans representing different stages of development (primarily the stages showing advanced spore formation) could be recognised even if live worms were examined microscopically, gently pressed down under a coverslip (Fig. 12, TR).

Description of the detected actinospore types

Most of the detected actinospore types were measured as described by Lom et al. (1997*b*). The main parameters of the actinospores found are presented in Tables 1–4.

Histological evidences

After fixation, histological processing and examination of infected worms selected in this way, actinospore parasites were found to occur first of all in the worms' gut epithelium and less frequently in their body cavity (Fig. 14).

Discussion

Primarily in recent years, actinosporean infection of oligochaetes has been studied successfully by numerous authors, among others by Mackinnon and Adam (1924), Markiw (1986), El-Matbouli and Hoffmann (1989), Yokoyama et al. (1991), El-Matbouli and Hoffmann (1993), Kent et al. (1993), El-Matbouli et al. (1995), Kent et al. (1995), Uspenskaya (1995), Yokoyama et al. (1995), and Trouillier et al. (1996). Most of the above-listed authors reported a relatively low (around 1%) prevalence of infection in worm populations. Yokoyama et al. (1993*a,b*) were the only investigators who detected > 4% actinosporean infection in *Branchiura sowerbyi* in a goldfish-culturing pond. In a survey of the actinosporean infection of oligochaetes in natural waters of Hungary, Székely (1989) and Pallós (1995) also found similarly low levels of infection. In contrast, in the present survey e.g. raabeia infection of *Branchiura sowerbyi* showed a prevalence of 98% in certain periods of the year, and in the warm months a triactinomyxon infection of 3% and 4% prevalence was recorded even in the *Stylaria* and *Nais* specimens, respectively, which were found to have the lowest level of infection in this survey. These values are markedly higher than those reported earlier by other authors. The outstandingly high values recorded by us, however, only partially mean that the level of actinospore infection is so much higher in the fish ponds surveyed than in other habitats. The observed difference can be attributed primarily to the different examination technique used by us. Today, it is already well known that a given oligochaete may be infected by

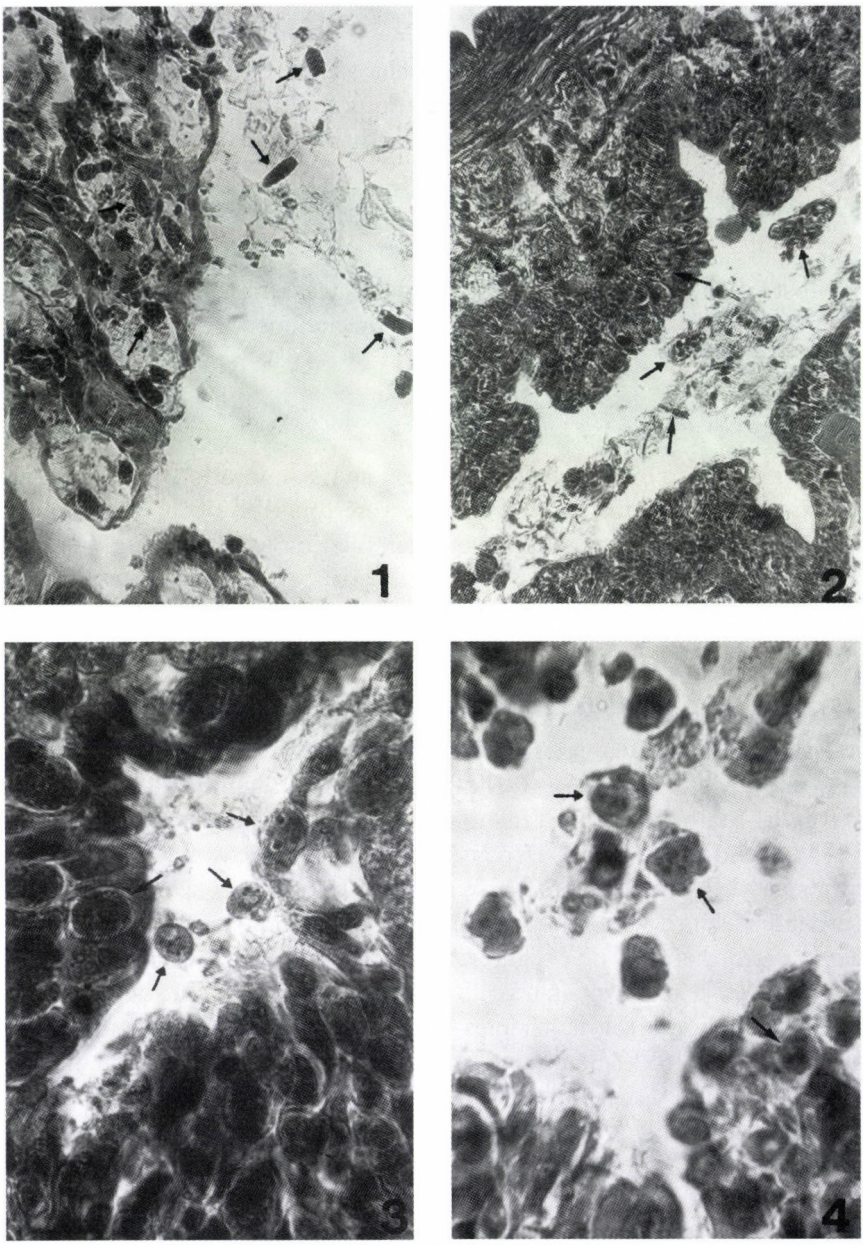


Fig. 14. Histological sections of actinosporean-infected oligochaetes; 1. Triactinomyxon infection in the gut of *Tubifex tubifex* (× 210); 2. Raabeia infection in the gut of *Branchiura sowerbyi* (× 250); 3. Aurantiactinomyxon infection in the gut of *Branchiura sowerbyi* (× 960); 4. Neoactinomyxon infection in the gut of *Branchiura sowerbyi* (× 1750); arrows: spores in the gut lumen and in the gut epithelium

actinospores representing different development stages while the mature spores will be excreted only after an about 3-month period of intra-oligochaete development. The fact that in this study individual oligochaete specimens were regularly examined over a period of about 3 months, greatly elevated the ratio of positive individuals and revealed that a single examination of any given worm cannot give reliable data on the prevalence of actinosporean infection, as it shows exclusively the ratio of worms that contain mature spores and spores being excreted at the given point of time.

The data obtained in this study indicate that actinospore infection of oligochaetes persists throughout the year and is characterised by seasonality manifesting itself primarily in the appearance of worms containing mature spores, which reaches its peak in the warmer months.

A further explanation of prevalence values far exceeding those reported in the literature may be that in the present survey the worms were collected from ponds in which several fish species were reared simultaneously in an intensive manner. As a result, the number of myxosporeans (and thus the actinospore forms) occurring there can be far higher as compared to the level of actinosporean infection of oligochaetes derived from ponds in which other fish species (salmonids, goldfish, catfish) are reared in monoculture (Pote and Waterstrat, 1993; Yokoyama et al., 1993*a,b*; McGeorge et al., 1997).

The different actinospore types could be found in both the smaller and the larger oligochaetes. Thus, triactinomyxons were detected in *Tubifex tubifex* of large body size just like in the smaller *Stylaria* and *Nais* species. It should be mentioned, however, that triactinomyxon types, which are so common in other oligochaetes, could not be detected in *Branchiura sowerbyi* showing the highest prevalence of infection. The actinospores found during the present survey were defined on the basis of the main taxonomic units (triactinomyxon, raabeia, aurantiactinomyxon and neoactinomyxon) described by Janiszewska (1955, 1957) and Marques (1984); however, we agree with the view of El-Matbouli et al. (1992), Kent et al. (1993), Yokoyama et al. (1993*a*), Kent et al. (1995), Yokoyama et al. (1995), Trouillier et al. (1996), and McGeorge et al. (1997), i.e. that these cannot be regarded as independent taxonomic units. The accepted names are used only for denoting the type of developmental stage of a given myxosporean species. The number of the 28 actinospore types detected by us roughly corresponds to that of myxosporean species hitherto found in the fish farm. It is likely, however, that some of the actinospores found during this survey had come from the inflow water, rather than being a developmental stage of myxosporeans parasitising fish reared in the ponds. While we do not venture to identify any of the detected actinospore types as a developmental stage of any of the myxosporean species parasitic in the ponds in question, based upon the experimentally proven developmental cycles reported in the literature (Wolf and

Markiw, 1984; El-Matbouli and Hoffmann, 1989; El-Matbouli et al., 1992; Benajiba and Marques, 1993; El-Matbouli and Hoffmann, 1993; Kent et al., 1993; Uspenskaya, 1995; Yokoyama et al., 1995; El-Mansy and Molnár, 1997) as well as our own work currently under publication (El-Mansy and Molnár, 1998) it can be assumed that triactinomyxons and raabeias represent the developmental stages of species belonging to the *Myxobolus* genus while neoactinomyxons and aurantiactinomyxons may be the developmental stages of species included in the genera *Myxidium, Zschokkella, Hoferellus* and *Thelohanellus.*

Differential diagnosis

On the basis of their dimensions presented in Tables 1–4 and their schematic drawings shown in Figs 1–4, the 4 triactinomyxon, 4 raabeia, 12 aurantiactinomyxon and 8 neoactinomyxon types detected during the survey relate to the types hitherto described in the literature as follows.

As this survey provided a picture of the actinosporean infection of oligochaetes derived from a fish farm culturing cyprinids, it is not surprising that the 4 triactinomyxon types found by us differ from the triactinomyxons detected by other authors (Hamilton and Canning, 1987; El-Matbouli and Hoffmann, 1993; McGeorge et al., 1997) in waters inhabited by trouts. Nor are these four triactinomyxon types identical with the triactinospore of *M. drjagini* and *M. hungaricus* described by El-Mansy and Molnár (1997, 1998) from a fish farm and from Lake Balaton, respectively. Although some of the dimensions (e.g. those of the style or the caudal processes) may be identical with the data reported by other authors, in these cases differences are found e.g. in the number of secondary cells located in the triactinospores.

As with the triactinomyxons, the 4 raabeia types found by us did not prove to be identical with the forms hitherto described in the literature (Janiszewska, 1957; Janiszewska and Krzton, 1973; Yokoyama et al., 1995; McGeorge et al., 1997). Although three out of the 4 raabeia types found by us (types 1, 2 and 4) have caudal processes similar in size to that described by Yokoyama et al. (1995) from *Branchiura sowerbyi* and, moreover, type 4 even has spore body parameters identical with those of the latter, type 4 described by us has much larger polar capsules than that reported by the above-cited Japanese authors. The other raabeia types included in our survey differ from those described by other authors in several parameters.

By their dimensions, none of the 12 aurantiactinomyxon types can be identified with the forms described by Marques (1984), Styer et al. (1992) and Troullier et al. (1996). Nor can the forms found by us be compared with the aurantiactinospores described by El-Matbouli et al. (1992), Grossheider and Körting (1992), and Benajiba and Marques (1993), as the authors did not give

spore dimensions in these works. Although the aurantiactinomyxon type presented by McGeorge et al. (1997) resembles the 12 aurantiactinomyxon types found by us in many of its parameters (spore cavity diameter, polar capsule dimensions and length of caudal processes), the caudal processes of the Scottish form widen out much more than those of the forms found by us. These two forms also differ in their habitat and alternative host. The form described by Yokoyama (1997) as the aurantiactinospore of *Thelohanellus hovorkai* closely resembles our type 12 also found in *Branchiura sowerbyi*, with only slight differences.

The eight neoactinomyxon types collected by us differed from the forms described in the literature by Janiszewska (1955), Marques (1984) and Yokoyama et al. (1993*a*) in several of their dimensions.

A paper providing general and consistent guidelines on the description of actinospores on their own or as the alternative form of myxosporeans has been published only quite recently (Lom et al., 1997*b*). This is why it is often difficult to compare the forms found by us with those described in earlier works. It is to be hoped that in the future all descriptions will conform to the general guidelines, which would make it easier to compare the results obtained by different researchers.

The main objective of the present work was to complement our knowledge of the myxosporeoses of fish farms with data on those myxosporeans' alternative forms living in oligochaetes. Our future goal is to assign the highest possible number of the detected actinosporeans to individual species of myxosporeans, with the help of experimental work.

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EFFECTS OF DIFFERENT HYBRIDS, STRAINS AND AGE OF LAYING HENS ON THE CHOLESTEROL CONTENT OF THE TABLE EGG

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Three experiments were carried out to investigate the effects of age, moulting and strain of hens on the weight, total cholesterol content (TCh) and cholesterol concentration (CCh) of egg yolk. Only slight differences were found in the TCh and CCh of eggs between white and brown egg layer hybrids (Shaver SC 288 vs. Shaver SC 579) of the same age. On the other hand, significant differences ($p < 0.05$) were found in yolk CCh and TCh of both hybrids during the production period. The change in yolk CCh was periodical: it decreased in both hybrids until 45 weeks of age, then it increased, reaching a plateau between weeks 51 and 54, and declined again at the end of production. TCh, however, increased until 51 (Shaver SC 579) or 54 (Shaver SC 288) weeks of age, which could be attributed to the steady increase in yolk weight throughout the production period. In the eggs of Tetra-SL hens yolk weight, yolk CCh and TCh were slightly higher after moulting (at 108 weeks of age) than in the first production period (at 48 weeks of age), but the differences were not significant ($p > 0.05$). Significant inter-strain differences ($p < 0.05$) were found, however, in the yolk parameters measured. The highest values were found in the eggs of New Hampshire hens, as compared to the traditional Hungarian breeds. The eggs of traditional pure-bred hens contained a substantially higher amount of cholesterol than those produced by the hybrids.

Key words: Cholesterol, egg, laying hen, strain, production level

Extensive research has been conducted in recent times to lower the cholesterol content of table eggs. This has been motivated by the significant correlation between coronary heart disease and fat composition especially of foods of animal origin (Sinclair, 1953; Parkinson et al., 1994).

In a study with various species of birds, Bitman and Wood (1980) found cholesterol values ranging between 15 and 25 mg/g yolk. In a related study, Bair and Marion (1978) measured the cholesterol content of eggs of guinea-fowl,

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domestic fowl, pheasant, quail, turkey, duck, goose and pigeon. They obtained the lowest value in the egg of guinea-fowl and the highest in that of pigeon. They also observed a significant difference in the cholesterol level of yolk between wild turkey and duck. In both species, the value of domesticated birds was higher. Chand (1980) also observed statistically significant differences between different breeds and strains of fowls in egg yolk cholesterol content.

The heritability (h^2) of egg yolk cholesterol content was found to be 0.21–0.26 (Cunningham et al., 1974; Washburn and Nix, 1974; Ansah et al., 1985). Others (Becker et al., 1977; Atalla et al., 1983) reported even lower values (0.04–0.18). In spite of these findings, Cunningham et al. (1974) classified a White Leghorn population into 3 groups based on the total cholesterol content (TCh) of their eggs and observed significant differences in the TCh of eggs produced by the following generation of the different groups. This suggests that in spite of the low h^2 values cholesterol content can be decreased also by selection.

Washburn and Marks (1985) showed significant differences in egg cholesterol content among different layer populations and among individual layers of the same population. This was also confirmed by the results of Edwards et al. (1960) and Beyer and Jensen (1992). In a study conducted on several Canadian farms with White Leghorn layers, Chung et al. (1991) reported cholesterol concentrations (CCh) of 12–15 mg/g yolk for commercial eggs. Significant differences were observed among different farms and also within individual ones.

Studying the relationship between egg shell colour and CCh, Cherian et al. (1990) reported an average CCh of 14.74 mg/g yolk for white-shelled eggs and 17.75 mg/g yolk for brown-shelled eggs. In contrast, using Shaver SC 288 hybrid layers (producing white-shelled eggs) and Shaver SC 579 hybrids (producing brown-shelled eggs) under similar feeding and management conditions, Angelovicová et al. (1994) obtained significantly higher levels of cholesterol in eggs produced by the Shaver SC 288 hybrid. They also investigated the eggs of New Hampshire layers raised on small farms under different feeding conditions. New Hampshire hens laid eggs with much higher CCh than the above-mentioned hybrid layers. On the other hand, Scholtyssek (1992) could not observe a significant difference in CCh content between white- and brown-shelled eggs.

TCh of yolk seems to correlate positively with the age of hens and with the weight of egg and yolk (Cunningham et al., 1974; Becker et al., 1977; Beyer and Jensen, 1989; Chung et al., 1991). Conflicting results were reported, however, on the change of CCh during the laying period. Brendl et al. (1979) reported that CCh declined with the age of hens. At the end of the production period they found the CCh about 25% lower than at the beginning of the laying period. Spencer et al. (1978) and Salogeanu and Bota (1981) failed to show such differences among layers of different age groups. Chand and Razdan (1976) obtained the highest egg yolk CCh in 13- to 14-month-old layers, while Gissel et al.

(1976) reported a significantly higher egg yolk CCh in young layers. Jiang and Sim (1991) studied White Leghorn layers from the beginning of the laying period up to the sixth month of production. They observed that yolk weight and TCh of yolk increased while CCh decreased during that period. Hall and McKay (1993) found a decrease in egg yolk CCh between 20 and 30 weeks of age and a constant value afterwards.

The cholesterol content of eggs can also be different in the first and second laying periods. Studying the effect of moulting, Nix et al. (1974) showed slightly higher egg yolk cholesterol values in eggs laid after moulting than in those laid before that. However, the differences were not significant.

The objective of this study was to investigate the effects exerted by the genetic constitution and age of hens on the cholesterol content of table eggs.

Materials and methods

Experiment 1

The effect of the laying period on TCh and CCh was investigated. Two commonly used hybrids, Shaver SC 288 (producing white-shelled eggs) and Shaver SC 579 (producing brown-shelled eggs), were kept under similar feeding conditions. The experiment was conducted at the experimental farm of the Georgikon Faculty of Agricultural Sciences, Pannon University, Keszthely. Eighteen-week-old layers were housed in battery cages and fed a monophased diet (Table 1) throughout the production year. Twenty eggs were collected randomly from each hybrid in different periods of production (Fig. 1). After measuring the egg weight, the yolk and the white of eggs were separated. Yolk samples were also weighed, then homogenised with a glass rod and analysed for cholesterol content by gas chromatography according to the method of Beyer and Jensen (1989). The following parameters of yolk samples were calculated: cholesterol concentration (mg cholesterol/g yolk) and total cholesterol content (mg cholesterol/yolk).

Experiment 2

Yolk weight, yolk CCh and TCh of eggs from the first and the second production phase were compared in this experiment, using a Tetra-SL layer population raised in battery cages at Kotkoda Ltd. Farm, Fót, Hungary. After the first, 46-week-long production period, forced moulting was carried out by feeding 1.5% of ZnO in the diet and by shortening the lighting period. The age of moulted hens was 108, while that of the controls was 48 weeks. Both groups of hens were fed the same diet (Table 1). Twenty eggs from each group were collected randomly. Yolk sample preparation and analysis of cholesterol were done in the same way as in Experiment 1.

Table 1

Composition and nutrient content of the experimental diets

Ingredients (g/kg)	Diets		
	Experiment 1	Experiment 2	Experiment 3
Maize	420.0	428.0	467.0
Wheat	305.0	155.0	220.0
Soybean meal (extruded)	–	–	20.0
Soybean meal (extracted)	70.0	160.0	113.0
Wheat bran	–	–	40.0
Wheat flour by-product	–	20.0	–
Sunflower meal (extracted)	40.0	40.0	–
Fish meal (defatted)	50.0	10.0	44.0
Meat meal	30.0	–	–
Blood meal	–	10.0	–
Alfalfa meal	–	20.0	–
Wheat-germ	–	50.0	–
DL-methionine	–	0.5	–
Calcium carbonate	72.0	90.0	83.0
Sodium chloride	3.0	2.5	3.0
Monocalcium phosphate	5.0	9.0	5.0
Vitamin and mineral premix	5.0	5.0	5.0
Total	1000	1000	1000
Calculated analysis			
ME (MJ/kg)	11.46	11.01	11.93
Crude protein (g/kg)	179.8	175.3	169.9
Crude fibre (g/kg)	25.8	33.4	29.3
Crude fat (g/kg)	28	30.7	32.3
Cholesterol content (g/kg)	0.5	0.1	0.3
Methionine + Cystine (g/kg)	7.2	7.4	6.3
Lysine (g/kg)	8.0	8.1	8.6
Linoleic acid (g/kg)	12.8	13.3	13.9
Ca (g/kg)	34.5	36.7	35.1
P (g/kg)	7.0	7.9	6.3

Experiment 3

Since no information was available on the cholesterol content of eggs of the different pure-bred hens kept in Hungary, the studies also covered the comparison of eggs from the following nine different strains: Hungarian Yellow, Hungarian Speckled, Hungarian White, Transylvanian Naked-neck White, Tran-

sylvanian Naked-neck Black, Transylvanian Naked-neck Speckled, New Hampshire White, Gödöllő Strain of New Hampshire. Hens of all strains were 44 weeks old, kept at the Small Animal Research Station, Gödöllő under the same system of management (deep litter system) and fed the same diet (Table 1).

Statistical analysis

Data were compared in all experiments by one-factorial analysis of variance according to Statgraphics version 5.0 (1991).

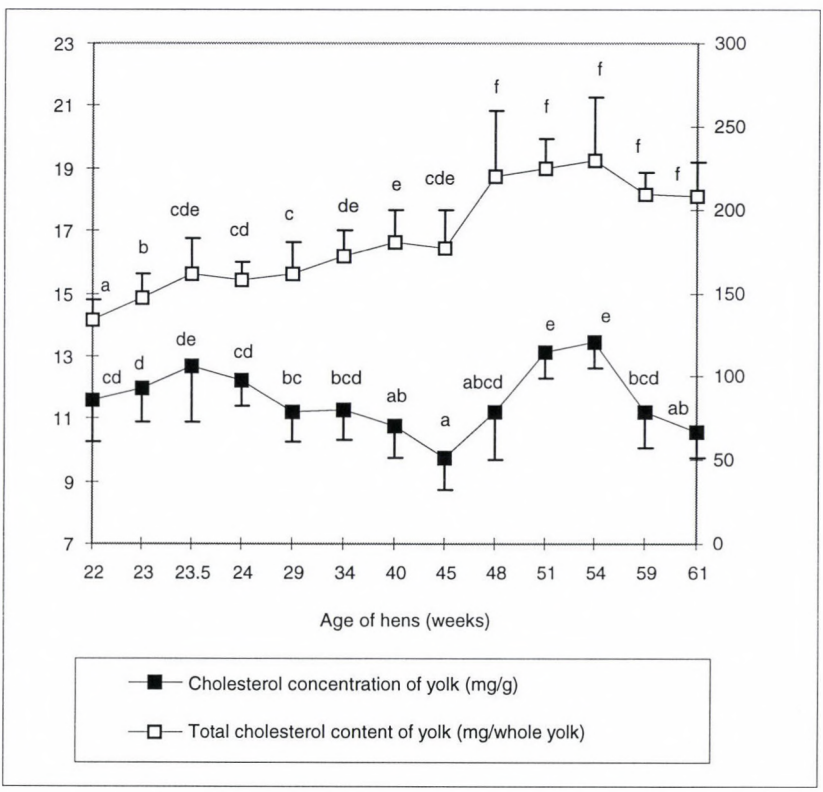

Fig. 1. Changes in yolk cholesterol concentration and total cholesterol content of the whole yolk during the laying period (Shaver SC 288). Squares represent mean ± SE. Means without common letters are significantly different ($p < 0.05$)

Results

Experiment 1

Changes in yolk CCh throughout the production period showed periodical patterns for both hybrids (Figs 1 and 2). Yolk CCh of the Shaver SC 288 population increased until 24 weeks of age, thereafter it decreased, reaching the lowest level (9.8 ± 1.1 mg/g) at 45 weeks of age. This was followed by a rise and the highest CCh (13.4 ± 0.8 mg/g) was found at 54 weeks of age. Thereafter cholesterol concentration declined again (Fig. 1). On the other hand, yolk TCh increased until 48 weeks of age, showing a higher plateau up to 54 weeks (229 ± 39 mg), and declined afterwards (Fig. 1).

A similar trend was observed in the changes of CCh for Shaver SC 579 hybrids, with the only exception that CCh declined during the first few weeks of production. The maximum value was obtained at 51 weeks of age (13.3 ± 1.6 mg/g), while the lowest (10.4 ± 0.6 mg/g) at 59 weeks of age. The tendency of changes in yolk TCh was very similar to that found for the hybrid Shaver SC 288 (Fig. 2).

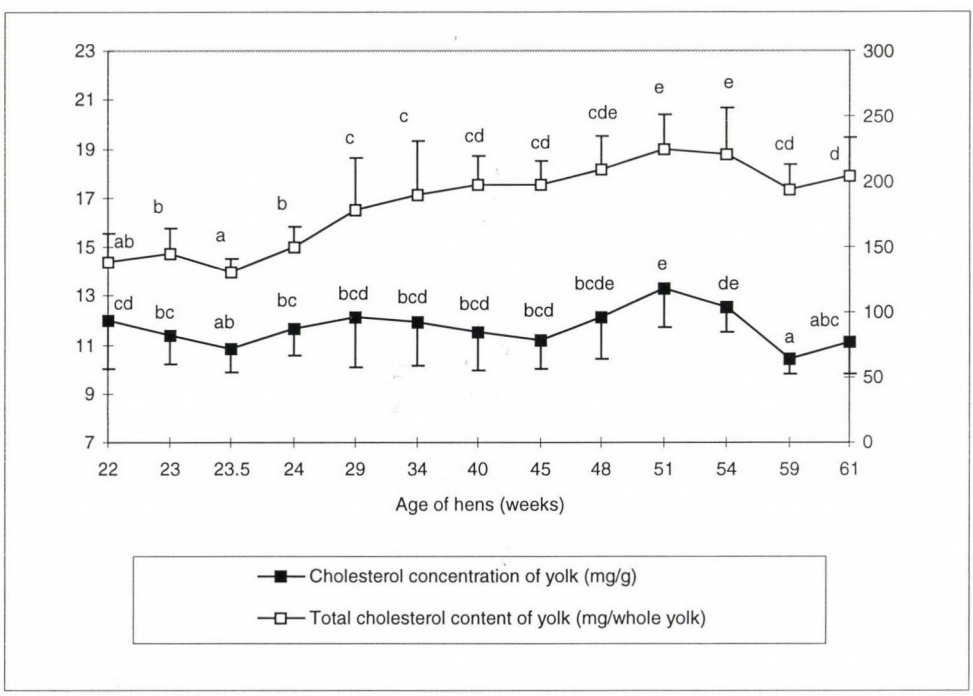

Fig. 2. Changes in yolk cholesterol concentration and total cholesterol content of the whole yolk during the laying period (Shaver SC 579). Squares represent mean ± SE. Means without common letters are significantly different ($p < 0.05$)

Comparing the yolk CCh and TCh of the two different hybrids at the same age, only slightly differences were found. Concerning CCh, the only significant difference ($p < 0.05$) between the two hybrids was found at 40 weeks of age, when the value for Shaver SC 579 hens was higher (12.1 ± 2.0 and 10.8 ± 1.0 mg/g for Shaver SC 579 and Shaver SC 288 hens, respectively). The opposite was found for yolk TCh. At 23.5 weeks of age, yolk TCh found for the Shaver SC 288 hybrid (161 ± 22 mg/yolk) significantly exceeded ($p < 0.05$) the value obtained for the other hybrid (131 ± 10 mg/yolk).

Egg yolk weight significantly ($p < 0.001$) increased with the age of layers, from 11.6 ± 1.2 g to 19.6 ± 1.3 g in Shaver 288, and from 11.6 ± 0.9 g to 18.4 ± 1.5 g in Shaver 579 hens.

Experiment 2

Slightly higher yolk weight (19.7 ± 0.9 g) was obtained after moulting when compared with the value obtained in the first production period (19.0 ± 1.7 g). The same tendency was found for yolk CCh (13.3 ± 2.4 mg/g and 12.7 ± 2.6 mg/g) and TCh (262 ± 44 and 240 ± 46 mg). The differences, however, were not significant.

Comparing the yolk CCh and TCh of Shaver SC 288, Shaver SC 579 and Tetra-SL hens at the same age (48 weeks), no significant differences were found, although the hens were fed different diets (Table 1).

Experiment 3

Significant differences ($p < 0.05$) were observed in yolk weight as well as in yolk CCh and TCh among the different strains studied in this experiment (Table 2). The two New Hampshire strains showed significantly ($p < 0.05$) higher values in all parameters, while yolk weight, CCh and TCh tended to be lower in the eggs of the traditional Hungarian breeds (Table 2).

Discussion

From the results of this study it appears that layer hybrid constitution, strain and age all have an influence on the yolk weight, yolk CCh and TCh of table eggs. Like in the studies of Angelicová et al. (1994), the biggest differences were found between the different strains in these experiments, too.

The change of yolk CCh was cyclic during the production period: both a decrease and an increase in yolk CCh were observed. Such a phenomenon has not been described yet. Gissel et al. (1976) also found a higher yolk CCh in younger than in older layers. In conformity with our findings, Jiang and Sim

(1991) also reported decreased yolk cholesterol concentration in the late phase of the production period. However, no researcher has recorded an increase in the late phase of production. The reason for the change of CCh during the production period is not known.

Table 2

Yolk weight, cholesterol concentration and total cholesterol content of whole yolk in different strains of laying hens

Strains	Yolk weight (g)	Cholesterol concentration (mg/g yolk)	Total cholesterol content (mg)
Hungarian Yellow	17.1 ± 1.8^{abc}	13.2 ± 1.3^{a}	224 ± 21^{a}
Hungarian Speckled	19.1 ± 1.9^{d}	12.9 ± 1.9^{a}	245 ± 36^{ab}
Hungarian White	18.1 ± 1.2^{cd}	13.9 ± 0.5^{a}	252 ± 15^{b}
Transylvanian Naked-neck White	16.1 ± 1.2^{a}	16.5 ± 2.9^{b}	265 ± 21^{b}
Transylvanian Naked-neck Black	16.5 ± 1.2^{ab}	13.8 ± 1.0^{a}	227 ± 21^{a}
Transylvanian Naked-neck Speckled	17.8 ± 1.8^{bcd}	13.8 ± 2.1^{a}	244 ± 30^{ab}
New Hampshire White	21.8 ± 1.3^{e}	16.9 ± 1.9^{b}	368 ± 53^{c}
Gödöllő Strain of New Hampshire	21.4 ± 1.7^{e}	16.6 ± 1.9^{b}	355 ± 49^{c}

Values represent mean ± SE. Means without common letters are significantly different ($p < 0.05$)

Yolk TCh was influenced mostly by the change in yolk weight. Egg weight and subsequently yolk weight increased with the age of layers. During the early production period the increase of yolk weight was more intensive than the decrease of yolk CCh. The steady increase of TCh in the first experiment can therefore be attributed to the increase in yolk weight rather than to the change in yolk CCh.

Regarding the effect of moulting, the present results support the findings of Nix et al. (1974) who also failed to find significant differences in the cholesterol concentration of eggs between the first and the second laying period.

Substantial differences were found, however, in the TCh of yolk between the different strains and hybrids. In the present experiments the lowest value (134 ± 12 mg) was measured in Shaver SC 288 hens at 22 weeks of age, while the highest one (368 ± 53 mg) in New Hampshire hens at 44 weeks of age. This considerable difference can already be important from the aspect of human nutrition. Since in Hungary New Hampshire hens are kept mostly on small farms under extensive conditions, these 'village eggs' can have a significantly higher cholesterol content than those produced by hybrid layers in an intensive system on large farms.

From the results of this study it can be concluded that factors other than nutrition can also play an important role in modifying the cholesterol content of the table egg. Of these factors, the genetic background and the age of hens have the greatest impact, which must be taken into consideration when judging the table egg from the point of view of human nutrition.

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CLINICAL RENAL ALLOGRAFT TRANSPLANTATION IN A DALMATIAN DOG: CASE REPORT

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A case of successful renal allograft transplantation performed in a two-year-old female Dalmatian dog suffering from end-stage chronic renal failure is reported. A one-year-old male German shepherd with severely injured spinal cord was used as kidney donor. Simultaneous kidney allograft transplantation combined with hypothermic initial perfusion as graft conservation was done, placing the donor kidney into the right iliac fossa of the recipient. The immunosuppression protocol consisted of prednisolone and azathioprine. Regular physical, laboratory, ultrasonographic and scintigraphic examinations were used for assessing both the morphology and the function of the allograft. After a two-week period of hospitalisation the patient was discharged in a remarkably improving condition. The recipient died on postoperative day 45 of respiratory insufficiency resulting from secondary pneumonia and pulmonary oedema. Neither macroscopic nor microscopic abnormalities of the allograft were revealed by necropsy.

Key words: Renal transplantation, dog, allograft, renal failure, renal scintigraphy

Renal allograft transplantation is a possible surgical approach in cases of end-stage chronic renal failure. It has been widely and successfully used in human beings for several decades. This therapeutic procedure has also been applied, with limited success, in dogs and cats in the USA and Canada (Gregory et al., 1986; Gregory et al., 1987). In 1995 a research project started at the Clinic of Surgery and Ophthalmology of the University of Veterinary Science Budapest, which aims at working out an entire diagnostic and therapeutic protocol for renal transplantation in dogs in Hungary. The 'best' candidates for the procedure are patients with irreversible chronic renal failure but still being in relatively acceptable general physical condition (Mathews, 1997). The potential donor of the allograft can be chosen from untreatable patients with central nervous system inju-

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ries (brain or spinal cord trauma) or from healthy, ownerless dogs, which are condemned to euthanasia (Németh et al., 1997). Immunological compatibility can be assessed by D-LA typing (Kelly, 1977) and by the MLR test (Kisken et al., 1969; Lu et al., 1991). Graft rejection (the histopathological process which is responsible for graft damage) must also be prevented by using specific immunosuppression: anti-thymocyte serum (Mathews et al., 1993), donor blood transfusion (Finco et al., 1985), and donor bone marrow transplantation (Mathews et al., 1990). Nonspecific immunosuppression includes glucocorticoids, azathioprine (d'Apice, 1984), cyclosporin A (Finco et al., 1987), and mizoribine (Minami et al., 1993). Surgical intervention consists of nephrectomy of the donor kidney, the conservation of the graft so that it can survive the ischaemic period, and the microsurgical procedure of venous, arterial and ureteral anastomosis. The main goal of postoperative care is to check the morphology and function of the graft constantly and systematically by physical, laboratory, ultrasonographic (Hricak et al., 1979; Pozniak et al., 1992; Nyland et al., 1997), scintigraphic (Németh et al., 1997) and pathological examination methods. If rejection occurs, the immunosuppressive protocol should be complemented by dexamethasone and supplementary intensive diuretic therapy (Finco et al., 1985). However, the outcome and clinical relevance of the renal transplantation used are always uncertain, and the owner should be aware of this fact.

Case report

A client-owned two-year-old female Dalmatian dog was brought to our clinic with the history of depression, anorexia, weight loss, vomiting and polyuria over a one-week period. Physical examination revealed poor condition, mild exsiccosis, prolonged capillary refill time, and urine-like odour of the breath. Both kidneys were found to be decreased in size on deep palpation of the abdominal cavity. Laboratory examination of the blood showed normal haematological parameters (red blood cells: 6.03 T/l, white blood cells: 11.9 G/l). The measured biochemical data were remarkably increased (urea: 52.1 mmol/l, creatinine: 659 μmol/l, phosphate: 4.0 mmol/l). Urinalysis demonstrated a mild proteinuria. Abdominal ultrasonography detected the characteristic signs of chronic nephritis (fibrosis) of unknown origin in both kidneys. The diagnosis was end-stage chronic renal failure. In view of the long-term ineffectiveness of any medical therapy, an attempt at clinical renal transplantation remained the patient's last chance to survive.

At that time, fortunately, a 1-year-old male German shepherd arrived at the clinic with an incurable thoracic vertebral luxation and complete paraparesis. The owner of this patient asked for euthanasia and, being informed about our re-

quest for renal allograft, willingly offered the organs of his dog for transplantation. Having regard to the urgency of the situation, the donor candidate immediately underwent a clinical physical, laboratory and ultrasonographic examination, which revealed normal excretory function. The recipient was placed in a separate ward of the hospital and received the starting immunosuppressive treatment (2 mg/kg azathioprine p.o. SID, 1.5 mg/kg prednisolone s.c. SID, 10 mg/kg amoxicillin and clavulanic acid s.c. SID) one day before the operation. The surgical procedure was carried out as a simultaneous transplantation on two surgical tables. First the donor nephrectomy was performed. After acepromazine (0.05 mg/kg s.c.), methadone (0.5 mg/kg s.c.) and atropine (0.01 mg/kg s.c.) premedication, anaesthesia was induced by ketamine (10 mg/kg i.v.) and maintained by halothane and oxygen. The donor received 70 IU/kg sodium-heparin i.v., then his left kidney was dissected out through a ventral midline incision so as to obtain as long portions of the renal artery, vein and ureter as possible. Before ligation and removal of the graft, the recipient was anaesthetised with propofol (4 mg/kg i.v.) followed by isoflurane and oxygen inhalation, then prepared for graft acceptance, being dissected in the right iliac fossa. Euthanasia of the donor was preceded by removal of the renal allograft with a long pedicle, after which it was subjected to a rapid conservation procedure called superficial cooling combined with hypothermic initial perfusion. The allograft was flushed with 4 °C heparinised lactated Ringer's infusion using a special vessel catheter inserted into the renal artery. As the kidney became anaemic (pale, greyish) and completely hypothermic, and after 70 IU/kg sodium heparin had been administered to the recipient i.v., an end-to-side anastomosis was performed between the common iliac and the renal vein followed by an end-to-end anastomosis between the external iliac and the renal artery in a single layer, using a two-point continuous technique with 7/0 USP polypropylene (Prolene-Ethicon) suture material. After removing the vascular clamps, checking the re-established renal circulation and controlling the minor haemorrhage around the anastomosis area, a modified 'drop-in' ureteroneocystostomy was done, using 5/0 polyglactin (Vicryl-Eticon). Ureteral peristaltic movements commenced one minute after declamping. Restarting of renal function was aided by acceleration of the i.v. infusion and by the administration of furosemide 2 mg/kg i.v. The allograft was stabilised in its place by nephropexy using two interrupted stitches sutured with 3/0 polyglactin (Vicryl-Eticon) between the perirenal connective tissue and the internal surface of the abdominal wall. The two, functionally altered native kidneys remained untouched. The abdominal cavity was closed routinely. On the day of surgery the dog received a repeated furosemide and infusion therapy, and she produced a small volume of bloody urine. Immunosuppressive therapy was continued in the dose described above, up to the end of the second week after surgery, when the dose of prednisolone was reduced to 1 mg/kg s.c. SID. In or-

der to evaluate allograft function, physical, laboratory, ultrasonographic and scintigraphic check-up examinations were done regularly. During the two-week-period of hospitalisation, the recipient was examined physically every day (general clinical impression, appetite, urination, defecation, body temperature, state of circulation and respiration, external palpation of the graft, state of the wound). Laboratory data (blood count, urea, creatinine, phosphate) were measured, and qualitative ultrasonography and ultrasonographic measurement of the renal volume were done five times. Renal scintigraphy (perfusion, renogram, graft size, T_{max}, $T_{1/2}$, MTT) was performed twice.

After a 2-day period of anorexia, depression, and spontaneous bloody urine excretion, the patient started to eat, her general state was improving and the urine became clear and straw-yellow. Palpation of the graft was painless and the surgical wound was intact. The body temperature remained in the normal range (mean: 38.8 °C). The most important laboratory findings are summarised in Figs 1–3 and Table 1.

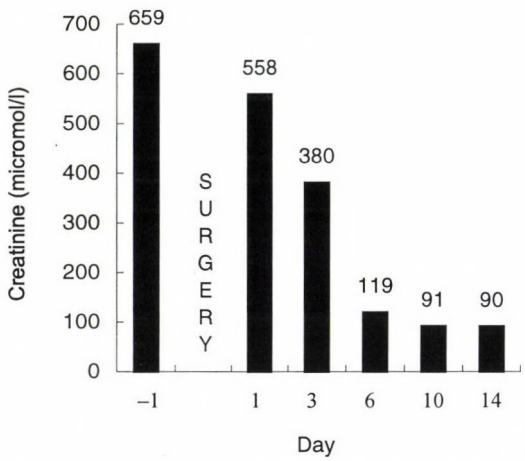

Fig. 1. Pre- and posttransplantation levels of blood creatinine

Ultrasonography revealed a slight increase in the size of the allograft on the first postoperative day. The structure of the kidney seemed normal with no expansion in the pyelon. The ureter was intact, the new orifice was found near the apical region of the urinary bladder. At the second examination (three days later) the size of the allograft decreased to normal. No further change was found by ultrasound up to the time of discharge from the hospital.

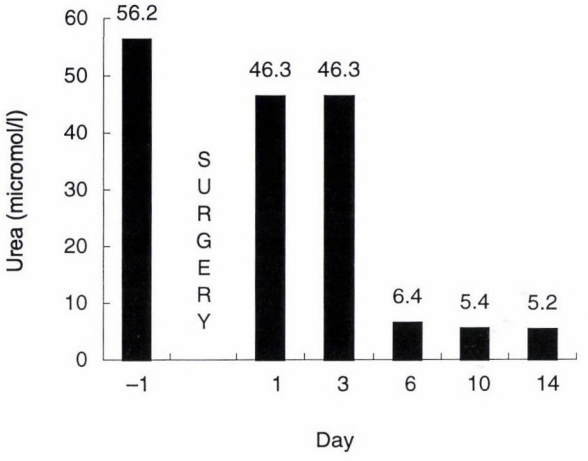

Fig. 2. Pre- and posttransplantation levels of blood urea

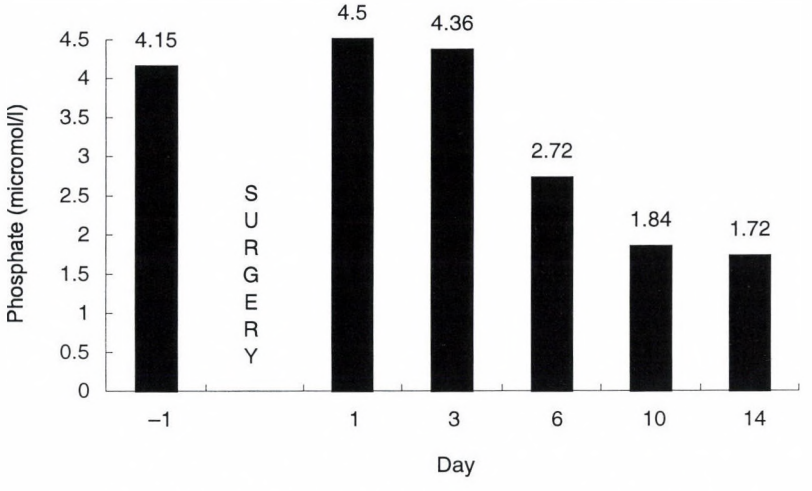

Fig. 3. Pre- and posttransplantation levels of blood phosphate

Table 1

Pre- and posttransplantation changes in the main parameters of the urine

	Day					
	–1	1	3	6	10	14
Concentration	1023	1015	1020	1028	1030	1030
Protein	++	++	+	+	–	–
Blood	+	++	++	–	–	–

An analogue gamma camera (Picker Dyna, 9011, Picker Co., USA) connected to a dedicated digital data analyser system (DIAG v 1.80) was used to perform ^{99m}Technetium L,L-ethylenedicysteine (^{99m}Tc EC) quantitative renal scintigraphy. The patient was anaesthetised with a combination of acepromazine (0.05 mg/kg s.c.) and ketamine (10 mg/kg i.v.). The radiopharmaceutical was applied in a bolus of 120 MBq/0.5 ml. The perfusion phase was evaluated in the first 60 seconds. Renograms were calculated between minutes 0 and 20. Quantitative data of renal morphology (size in pixels) and function of the remaining original kidneys as well as the graft (T_{max}, $T_{½}$, Mean Transit Time = MTT, relative renal function) were determined and evaluated. The results of scintigraphy are illustrated and detailed in Figs 4–5 and Table 2.

Table 2

Quantitative data of ^{99m}Tc-EC renal scintigraphy one week after transplantation. Note the considerable differences between the characteristic parameters of the transplanted graft and the original kidneys

	Sizes (pixels)	T_{max} (min)	$T_{½}$ (min)	MTT (min)	relative kidney function (%)
Left kidney	236	7.000	14.723	27.426	8.1
Right kidney	242	7.000	16.874	34.516	7.2
Transplanted kidney	357	3.666	6.674	14.089	83.7

In view of the definite improvement in the patient's general and excretory status, the recipient was discharged from the clinic at the end of the 2nd postoperative week. Azathioprine and amoxicillin were continued according to the protocol with a reduced prednisolone dose (1 mg/kg p.o. SID in week 3, 0.67 mg/kg p.o. SID in week 4, and 0.5 mg/kg p.o. SID thereafter). Isolated keeping and careful handling were recommended to the owner to reduce the risk of secondary infection. Any change in the animal's general status was to be reported to the clinic.

During the subsequent several weeks the patient ate and drank well, gained 6 kilograms in weight, and completely rehabilitated in the family's life. On postoperative day 43 the owner reported by telephone a rapid deterioration in the dog's appetite and a fever of 40 °C. The dog developed a severe respiratory disorder characterised by coughing and dyspnoea. Despite medical treatment with enrofloxacine and adequate intensive therapy including infusion, furosemide and diaphylline, the Dalmatian suddenly died on postoperative day 45. Necropsy revealed a respiratory insufficiency caused by severe purulent pneumonia and pulmonary oedema. Morphological disorders or signs of rejection were not found in the allograft.

Discussion

The clinical allograft transplantation was attempted in a Dalmatian suffering from end-stage chronic renal failure. As a recipient, the patient seemed to be 'ideal' regarding its relatively acceptable general condition and the moderately elevated laboratory values (Mathews, 1997). The donor selection was incidental, having a severely injured incurable dog suffering from irreversible spinal cord lesions. The MLR test should have been done in order to properly assess the expectable prognosis (Lu et al., 1991). Nonspecific immunotherapy included the administration of prednisolone and azathioprine, which was started one day before surgery (Finco et al., 1985). The use of cyclosporine was not possible as the owner could not afford it. The surgical intervention was performed by the conventional technique widely published and recommended (Gregory et al., 1987; Mathews, 1997). After some initial difficulties with restarting allograft function, the patient was saved from uraemia, which was confirmed by physical, laboratory and supplementary ultrasonography and scintigraphy. The most important and informative physical sign of rejection to be checked is painfulness of the allograft on palpation, which was not experienced during the examination period (Finco et al., 1985). Blood urea, creatinine and phosphate gradually returned to the normal levels by the sixth day after surgery. The main parameters of urine (concentration, protein) were normalised by postoperative days 6 and 10, respectively. The blood content of urine disappeared within a few days. Ultrasonography revealed a mild increase in the size of the allograft immediately after transplantation. That was a normal reaction to elevated blood pressure caused by the greater diameter of the 'new' nutritive vessel (i.e. the external iliac artery) of the renal artery (Nyland et al., 1997). There was no evidence of any rejection episode during hospitalisation. The advantage of scintigraphy performed with ^{99m}TC EC as radiopharmaceutical excreted via tubular secretion (Németh et al., 1997) was the precise demonstration of graft function by static as well as dynamic perfusion and renogram images. The main disadvantage is the need to 'immobilise' the patient, which makes this diagnostic procedure risky. At the end of the second postoperative week the patient was discharged at the owner's request in a stable posttransplantation condition. Rapid deterioration in the recipient's condition might have resulted either from the dog's immunosuppressed state or from her outdoor keeping.

Although the survival time was relatively short, the overall management can be considered successful in view of the allografting procedure and the reestablishment of excretory function. At the same time, this case demonstrates the potential risk of secondary infections that can markedly alter the outcome of renal transplantation.

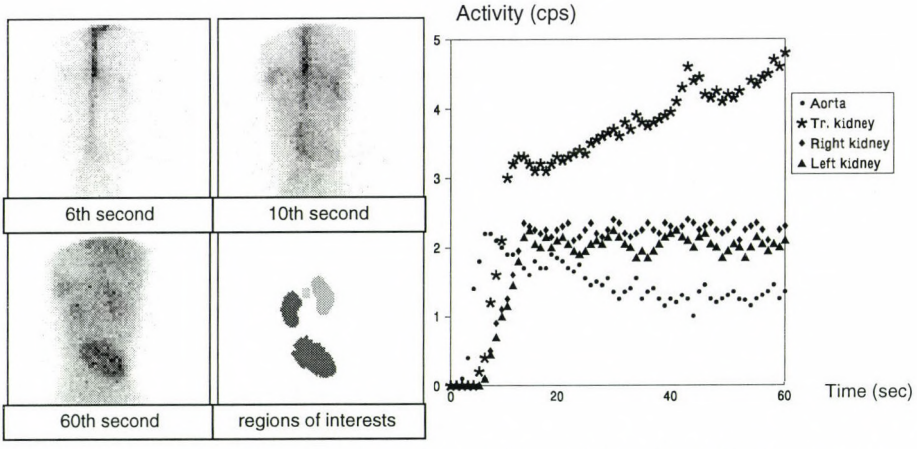

Fig. 4. Static images and time-activity curves of the perfusion phase (0–60 sec) one week after transplantation. Sixty '1-second' frames were taken after 120 Mbq/0.5 ml 99mTc-EC injection over the abdominal-pelvic region. Note the faster and higher activity peak of the graft versus the original kidneys. The dynamic study of the perfusion phase also indicates a much better blood perfusion of the transplanted kidney than that of the original ones

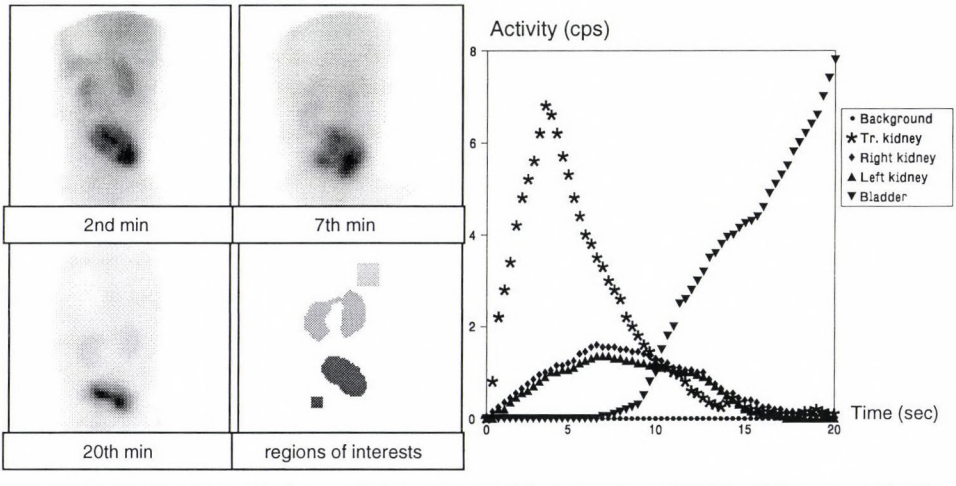

Fig. 5. Static images and time-activity curves of the renogram (0–20 min) one week after transplantation. Sixty '20-second' frames were detected by the renogram study between minutes 0 and 20. The transplanted kidney shows normal renogram pictures confirmed by physiological renogram curves with good perfusion, excretion and drainage. The study of the two original kidneys reveals delayed and low-peaked renogram curves indicating abnormal perfusion and excretion

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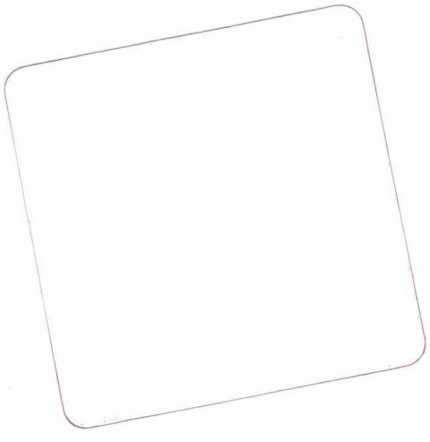

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Plenary Lectures of the 4th International Deer Biology Congress



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Preface

It is our hope that, following the success of the first three occasions on which the International Deer Biology Congress was held, the fourth IDBC to be organised in Hungary will also provide researchers and specialists in the practical field with an outstanding opportunity to become acquainted with a wide range of the most recent findings emanating from research projects involving wild or farmed deer. A further objective is to enable the approximately 150 participants from 29 countries to exchange practical experience and observations, and also to provide a forum for the establishment of both professional connections and personal acquaintances with others engaged in similar professional fields.

On the recommendation of the international steering committee, the scientific programme of the congress and the designation of the eight sessions into which it is divided follow the traditions already established. It was the wish of the local organising committee to create the opportunity for the inclusion of certain specific subfields suggested by participants; therefore, in addition to the Antler Symposium, eight other workshop topics can be seen to encompass the scientific programme of the congress.

In the interest of ensuring that the content of the plenary presentations introducing the respective sessions, to be given by internationally recognised researchers, should be available in an up-to-date form for wider scale dissemination, the speakers were requested to submit their manuscripts for publication in this issue, to be available not only to participants of the congress but also to readers of specialist literature in this field anywhere in the world.

Our thanks are due to the scientists of international repute who have undertaken to deliver the plenary lectures opening the sessions. We would like to convey our particular thanks to those who submitted manuscripts in advance at our request, thus enabling us to make the content of their presentations available to congress participants prior to the sessions. In order to disseminate the findings and observations of these outstanding scientists as widely as possible, we have also published the text of their lectures in the journal *Acta Veterinaria Hungarica*, volume 46, issue 3 (1998).

We owe thanks for the opportunity to publish this material primarily to the authors of the papers included. Acknowledgements are due to the Editorial Board of *Acta Veterinaria Hungarica*, and to András Székely, managing editor of *AVH*, for his work in the editing of this publication. We would also like to thank the sponsors of the congress for their generous support.

The Organising Committee will be gratified if readers find the results and observations contained by this publication to be of use, and to be suitable for application in their everyday work, since this would constitute the highest form of recognition of the work of the authors.

Kaposvár, June 1998

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Plenary Lectures of the 4th International Deer Biology Congress

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MANAGEMENT OF INDIGENOUS NORTH AMERICAN DEER AT THE END OF THE XXTH CENTURY IN RELATION TO LARGE PREDATORS AND PRIMARY PRODUCTIVITY

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Five deer species occupy North America: caribou (3.6×10^6 individuals), moose (1.1×10^6), white-tailed deer (28.5×10^6), mule deer (5.0×10^6) and wapiti (1.1×10^6). Caribou characterise the north of the boreal forest and the tundra, whereas moose dominate in coniferous and mixed forests growing further south. White-tailed deer are typical of the deciduous forests of the east while mule deer replace them in the mountainous terrain of the west. Wapiti possess the smallest range, mostly adjacent to the prairies to the west. The two specialised deer predators show a reduced distribution: wolves are almost restricted to Canada, and cougar to the mule deer range. We determined the current status of each species with the help of a questionnaire mailed to all jurisdictions harbouring deer. Most reports of threatened populations concerned caribou whereas many jurisdictions declared overabundance of white-tailed deer and wapiti. Hunting was allowed for all species when they abounded in a jurisdiction. Hunters harvested annually 7.0×10^6 deer on the continent, 87% being white-tailed deer. The two species that caused most conflicts with humans had the highest harvest rate: 16–17%. In terms of biomass, white-tailed deer and wapiti yielded the highest harvests, with 55 and 39 kg $\times$ km^{-2} of range, respectively. The average standing biomass of deer in winter ranged between 28 kg $\times$ km^{-2} in Nevada to 901 kg $\times$ km^{-2} in Indiana. The lowest standing biomasses occurred in the boreal forest (predators), in the prairies (agriculture) and in the south-west (aridity), and the highest ones, in the south-east, although only white-tailed deer is present. The current abundance of deer in North America parallels, in general, the primary production of the landscape, but predators and human activity modify this pattern.

Key words: Deer species, caribou, moose, white-tailed deer, mule deer, wapiti, North America, abundance, management, predators, hunting, harvest, standing biomass

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ECOLOGICALLY SOUND MANAGEMENT: ASPECTS OF MODERN SUSTAINABLE DEER FARMING SYSTEMS

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Modern deer farming systems have become increasingly intensive allowing strategic feeding for production and genetic improvement programmes. Meeting feeding standards that account for changing nutritional demands related to seasonality and reproductive state is critical. As the industry matures there is a growing awareness of the balance between retaining natural behaviour in producing breeding stock on larger extensive holdings and intensification systems for performance in young stock. Stocking rates are critical determinants of success as land use and capability needs are matched with an increasing stratification of stock type and purpose. Food product safety and welfare considerations of farmed deer are being driven by consumer demands. Farm quality assurance and codes of practice are developing to ensure that deer farming meets and exceeds international expectations of land use and deer welfare in modern deer farming systems.

Key words: Farmed deer, land use, feed requirements, sustainability, management

Within the last 30 years modern commercial deer farming systems have developed, or are emerging in most agriculturally advanced countries throughout the world. Such development is also paralleled by new technology and management refinements in the well established predominantly pen fed deer in China (Quin, 1996) and the huge extensive rangeland enclosures of Russia (Wallis, 1993).

Production systems have either expanded on traditional game ranching via some variant of controlled rangeland management, or more commonly have been adapted from intensive pastoral farming techniques taking into account the innate behaviour and biology of deer.

This 'continuation of exploitive strategies' as defined by Hudson (1989) recognises that there are no rigid boundaries between systems of ranching, herding or hunting, but farming tends to focus on smaller units of valuable and productive agricultural land where fencing costs are high. Issues of economic efficiency; through cost of production, value of products, opportunity cost and entrepreneurial return, technology cost and the desire for achieving some status of excellence does impact on animal/herd well-being and production sustainability.

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Modern deer farming systems almost invariably impose artificial constraints on herd structure through well intentioned but not necessarily well informed application of traditional livestock farming practices.

Many aspects of deer farming require knowledge and adaptation of the response of the wild deer population to farming intervention in any form. This particularly applies to sustainable utilisation of the farm ecosystem and animal species (Benson, 1992), disease risk (Geist, 1992), and behavioural and social structure (Challies, 1985).

The base requirements for successful management of farmed deer have been elegantly summarised by Haigh (1992) and Haigh and Hudson (1993). Deer farm systems irrespective of scope, demand the integration of three key factors: (1) choice of appropriate production characteristics of the deer species that allow domestication; (2) an active management role in confinement, handling, nutrition, reproduction and disease prevention; (3) farming knowledge of innate deer biology and behaviour.

Again as Haigh (1992) defines, there is often a basic conflict in approach between extensive management and increasingly intensive farming. Extensive farming with low input preserves the natural biological and production features of deer and will certainly appeal to the market or to consumer perception. However, the trend to intensification, apart from the economic advantages in land development, allows planned pasture management for maximum production in relation to sex, age and deer species. Additionally intensification allows the opportunity to handle and select individual animals or groups for the productive purposes of breeding, slaughter or antler harvest (Fennessy et al., 1993).

For the purposes of this discussion the focus is on several farming systems and the apparent conflict between ecologically sound management and sustainable deer farming as an agricultural enterprise. Modern deer farming systems essentially have as their primary objective profitable commercial return based on products of venison, velvet antler and co-products (Fennessy and Taylor, 1989). In many instances achievement of commercial productivity is fuelled by demands for high value and scarce capital breeding stock. In the emerging industries this is particularly so when external sources of feral stock are limited by scarcity or by public opposition to wild capture — clear contrast to the rapid expansion in New Zealand (Elworthy, 1993).

It is not coincidental that the rapid advance of deer farming in countries like New Zealand, Australia, Canada and the UK have attracted considerable innovation in adapting existing farming systems utilising the observation and knowledge of behaviour in the wild. As the industry evolves, more attention is now being paid to the ecological impact of deer farming in terms of animal and land welfare, product image and sustainable land use.

Farmed deer production

(1) Herd management

The majority of farmed deer world-wide represent the Cervinae subfamily of temperate/cold zones and are markedly seasonal in their behaviour (Lincoln, 1992). In general such specific reproductive seasonality has a direct relationship to the available supply of food in the natural preserve. This parallels the usual farm pasture production curve and typical pastoral land management. While this broad supply/demand matching is important from the viewpoint of treating deer management as a variant on traditional animal husbandry, it is immensely variable in deer farming situations. More critical is an understanding of the endogenous seasonal rhythm of the animal and meeting the annual nutritional requirements accordingly within the farming system. Management techniques logically follow. Irrespective of the product stream of commercial deer farming there are basic management control procedures imposed on a deer herd to accommodate the differential production of age and sex classes within herd. From this distinction efficient farming begins to develop from changing land management systems for deer.

Males are separated from females either at weaning or at a pre-pubertal age of 12–15 months. Weaning marks the first point for sale or culling based on an objective or subjective classification of economic worth or genetic potential.

Pubertal separation marks an obvious sale or harvest point, but is also the first imposed intervention for breeding programmes. Significantly this time highlights a critical weight threshold point which impacts on breeding success and reflects the management of the young stock to date (Hudson et al., 1991; Asher, 1993).

Management further separates females and males by age and breeding status. First calving hinds are mated and managed separately from older stock. Antler producing stags are run separately by age groups for differential feeding and management in response to behaviour and timing of antler growth. Farming systems therefore are required to develop stock-proof subdivision and the basics of a broad rotational grazing systems for each stock class.

(2) Nutritional requirements

Because of the major differences between the three basic production systems (breeding hinds, velvet stags, and finishing and growth of young replacement stock) differing feed supplies are needed (Nicol, 1996). This may extend to developing specialist pastures or crops within the property (chicory, brassicas, and legumes/clover dominant pasture) (Kusmartono et al., 1995; Fennessy and Milligan, 1987) and variable management over the year.

Both the age and sex groupings and the application of specialised deer farming system to a particular land type have evolved from a management need

albeit as a legacy from other livestock farming practices, but does recognise the differential feed requirements over a year for each stockclass, and the need to develop a separate feeding regime. Failure to meet feed requirements, particularly through times of environmental stress such as severe winter or summer drought conditions, ultimately predisposes to disease risk (Griffin, 1989) or severely limited production via challenge to immunocompetence (Griffin et al., 1992), particularly yersiniosis, tuberculosis and Malignant Catarrhal Fever (Mackintosh, 1992*a,b*).

The application of knowledge of the seasonal feed requirements of deer has become the cornerstone of farm production (Fennessy et al., 1981; Hudson, 1998) and established management systems to overcome barriers (Suttie et al., 1987; Drew, 1996; Nicol, 1996).

Many deer farmers have readily adopted daily energy requirements in megajoules of metabolisable energy (MJ of ME) as the expression of deer dietary needs. It is now recognised at least in the pastoral situation that feed crude protein content of 14–16% is sufficient for maintenance and growth and protein sources in excess of 18% are expensive and to some extent wasteful unless used in strategic supplementation for velvet growth or for enhancing accelerated winter growth in young deer in housed or pen fed situations (Webster et al., 1998).

Strategic supplementation is at its most refined in the penned velvet antler herds of sika and malu deer in China and wapiti in Korea (Liang et al., 1993). Strategic feeding for velvet antler growth varies protein and energy within the diet through changing roughage and concentrate proportions according to the stage of antler growth. In the pastoral situation meeting the seasonal ME requirements is the most important nutritional objective. For pen fed animals female feed requirements for supplemental concentrates are advised at 17% early and mid-gestation, 19–20% late gestation and 21–24% during lactation.

Deer farming systems

Farming systems are becoming increasingly stratified according to land capabilities and climate. Four of these systems will be discussed.

(1) Ecotourism and trophy hunting

Large tracts of natural mountain terrain have been recently enclosed for controlled hunting and leisure activity in parts of Australasia. This is in keeping with the marketing value of a clean and natural image. While the environment remains very sensitive particularly to overstocking, this former feral range for introduced deer species represents the most natural form of modern deer man-

agement. Unlike managed hunting herds of Europe (Bubenik, 1989), most trophy animals are selected from external breeding studs on an annual basis. Stags are selected at the peak of their mature antler production and released for one or two seasons until harvest often without any breeding role or competing stock. The animals are often scored for trophy potential prior to relocation and priced accordingly with the added advantage that genetic merit is preserved via semen collection on the farm of origin. This market is very competitive with the desire to create fair chase hunt, trophy quality is high, and stock numbers are very low for a limited season. The rangeland provides more than adequate food such that regeneration of tussock and bushlands is encouraged. Problems of soil depletion through erosion and overgrazing are avoided. The ecotourism experience demands that containment fencing is not obvious, but requires substantial capital resources for adequate disease control and security and for the hosting and hospitality services that accompany the experience. With the recent introduction of elite breeding stock from the best deer herds of Europe, trophy stock has reached world record status although this is more a function of intensive high technology breeding programmes and farm nutrition, rather than a feature of the trophy park *per se*.

It is not uncommon to have access to the full variety of fallow, red deer, wapiti and introduced chamois combined with the opportunity for other outdoor sports and relaxation. Sheep and cattle grazing in this sensitive environment ceases which is in keeping with image and economics. Sustainability is based on trophy antler ranking and success in record books for the top operators in a fiercely competitive market.

Because of New Zealand's demanding disease eradication and surveillance schemes, particularly for bovine tuberculosis all animals have accredited free status on release and inspection protocols after harvest provide annual monitoring with no live animals removed from these enclosures unless passing full testing. In the absence traditional livestock, young deer on a full range of natural dietary options achieve high health and productivity status.

(2) Extensive breeding — hill and high country

Increasingly advanced operations in New Zealand are returning to very large breeding units in mid-high country terrain that after an initial high expense of fencing and broad subdivision are managed on low input cost, low stocking rate, low labour input basis. High numbers (up to 5000 hinds) in large groups are overwintered on improved native grasses often augmented by oversown legumes and improved grasses with valleys and flat areas utilised in a slow improvement programme with brassica crops and annual high producing autumn/winter grasses. The country's strength is in late spring and summer pasture production, adequate rainfall and scope so that low stocking rates allow natural hind herd

behaviour, optimum calving and weaning success without any major impact on pasture replenishment over the season. Overwintering costs are restricted to breeding hinds with a comparatively low feed requirement once weaning and mating rising plane of nutrition have been achieved. Larger properties use ultrasonic pregnancy scanning as a management tool to ensure only productive hinds are retained throughout the winter and calving period. Large mob sizes over winter do have the capacity to create localised overgrazing and some erosion particularly in newly cultivated land. Other costs involve routine handling of deer and because of the large numbers involved the facilities tend to be large and expensive for relatively little usage.

A management conflict can arise. Low stocking rates and complex contour, cover and terrain which allow for the most natural expression of hind herd behaviour particularly during parturition and mating invariably leads to poorer quality pastures for lactation and a loss of the tractability and quietness of breeding stock and their progeny. There is increasing evidence (Asher, pers. comm.) that excessive stocking rates (5 red deer hinds/ha) during parturition have a powerful impact on postnatal calf survival. Constant disruptions to dam/calf bonding profoundly influence true parentage and can add to calf wastage losses via mismothering and starvation. Asher and Adam (1985) recorded neonatal calf losses in intensively farmed red deer of 10–15% and a little higher in fallow deer. In a more extensive environment hinds have the opportunity to separate from the herd and calve undisturbed with little disruption and indications are that weaning rates increase although arguably at the cost of calf liveweight at the time sale. Infrequent handling can lead to problems in working with some individuals in the yards which leads to culling on temperament irrespective of production merit. Low input costs are also reflected in high health status as the herd has ready access to native forage and browse. Such situations tend to suit red deer rather than elk. Success is ultimately dependent on not overstocking the resource and utilising an appropriate winter system to cater for the climatic difficulties of largely inaccessible terrain.

Variants on this theme exist in rusa (*Cervus timorensis*) herds in New Caledonia and are developing in Australia. The introduced rusa present an interesting study within a developing industry where after accidental liberation (1870) the success of feral propagation rapidly reached pest proportions with devastation of crops and cattle pasture.

Greatest concentrations of deer (> 80/km^2) are found in arid zones abandoned by stock rearers where nutrition was maintained with fodder trees (*Leucaena leucocephala* and *farmesiona*) and natural grasses. Since farming was established in 1987, seasonal drought has forced farmers to adopt an emergency feeding strategy utilising irrigation, provision of green feed and expensive concentrates. Animal loss was prevented but the cost was uneconomic. The cost of rear-

ing deer on improved pasture was a factor of 10 times that on the natural environment (Le Bel and Dulieu, 1993). Rusa in this predictable rainy and dry season climate have developed an environmentally controlled breeding pattern in the extensive rangeland with peak sexual activity in winter and spring allowing calving after 250 day gestation to broadly coincide with the rainy season forage surplus (Bianchi et al., 1998). Intensive farm management is now limited to lactating hinds and growing young stock. Natural runs are reserved for breeding stock to utilise their grass-roughage dietary preferences and for shelter during cyclones. The integration of two radically different feeding and management systems in response to a consistent drought/rainy season situation underpins the successful linking of animal feeding needs to a sustainable feed resource. Natural runs are also effectively improved by planting and managing the grazing of fodder trees, stocking rates are effectively managed by strategic culling and trophy selection. Input cost of fence containment like the trophy park situation, is relatively low compared with the ongoing farm costs of the productive irrigated land.

(3) Intensive developed hill and downlands — breeding and velvetting units

This predominant New Zealand deer farming system can represent the best of deer breeding and production but is also the major source of production wastage and need for management improvement (Audige et al., 1993). Deer farming is often an additional diversification on highly developed and improved pastures where land maintenance requires considerable input of fertiliser, pasture renewals and subdivisional fencing for grazing control and stock management. Stocking rates are invariably high on an annual basis and require considerable skills for appropriate breeding and feeding strategies and animal health and welfare programmes.

Reproductive success is vital to the economic viability with a management focus on early weaning, condensed and finite calving patterns and a planned annual turnover of all young stock except replacements. Culling and sale criteria are closely related to level of performance, most particularly as a reproductive success right through to weaning. Spring-summer growth conditions are critical to the economic and functional success and while deer can be adequately supplemented through summer drought the ongoing effects on subsequent breeding and over-wintering have long term impacts on economic viability at the high stocking rates carried. Many farms have experienced functional problems of soil erosion through poorly located fence lines that increase behavioural unrest in an artificial herd structure and this can lead to unsustainable production. Red deer particularly pace fence lines and the combination of access to waterways, lack of shade and natural cover and relationship of hinds to young stock or large groups of non-breeding males during the rut all contribute to land damage, soil loss and

pasture depletion. In improved pasture situations deer will graze heavily on highly preferred legumes and varieties of herbaceous grass leading to unbalanced pasture swards dominated by less preferred species with a constant decline in production.

Problems of subdivision, and developing a more natural environment at least in part are being addressed through the New Zealand Deer Farmers Association quality assurance scheme (NZGIB, 1995) — essentially a Code of Practice that balances animal welfare and behavioural needs with sound management approaches. Most species of trees generally do not survive under sustained grazing pressure unless well protected, but planned planting and siting of tree blocks around waterways and along fence lines creates a visual barrier between groups. Re-siting of fence lines along crests rather than across the contour give flexibility in managing high stocking rates without affecting pasture quality management. With such intensification, deer become extremely docile for yarding and handling in health programmes, selection work, multiple handlings needed for reproductive technology events and in trucking for sale or slaughter.

Because hill farms are under intensive management the full expression of the genetic variety and ability to cross breed between North American and European strains of Cervids is also seen. The base New Zealand herd is rapidly evolving its own unique productive character as farmers select and inter-breed a variety of European strains of deer (Pearse and Goosen, 1998). Primary objectives are increasing body size with an ability to conceive earlier, improving growth rate and increasing velvet antler production. Breeding and selection for velvet has lifted production by up to 40% within one or two generations. This new merged deer strain will form the nucleus of the fully domesticated farmed deer and awaits now only the refinement of appropriate DNA technology to identify quantitative trait loci and polymorphic alleles that directly enhance quantitative production including those for disease resistance, venison growth, muscle yield and antler production (Tate et al., 1997; Tate et al., 1998).

While the diversity of such intensive farming is immense world-wide, the critical issue for sustainability remains determined by the fact that this land is primarily suited to animal production rather than cropping, and is limited by stocking rate, summer climate and economic viability.

(4) Finishing properties — fertile arable foothills and plain lands

The purchase and relocation of weaner deer which are 3–4 months old from larger breeding units to specialist finishers who have highly fertile arable land and have adapted pastures, crops and management to maximise animal growth is a major part of the New Zealand industry (Pearse, 1995). Similarly, in the UK (Fullerton Smith, 1993) and Denmark (Vigh-Larsen and Davies, 1998)

where the product is venison, intensive indoor and outdoor feedlot finishing systems are common features as deer farms increase in size. Winter pen feeding is also growing in popularity in the colder and wetter parts of New Zealand where existing sheep handling facilities are readily adapted and may also be used for photoperiodic manipulation to increase winter growth rates.

These systems are very much attuned to input costs and efficiency of production that allow young animals to reach ideal slaughter weights quickly and form the basis of contracted supply for chilled or fresh venison (e.g. Cervena^{TM}) fulfilling the market demands for consistency and continuity of supply. Producers face the major constraint of winter depression in deer growth rate. Research has assisted in developing systems to minimise the effects of this natural growth depression (Suttie et al., 1996*b*; Vigh-Larsen and Davies, 1998; Webster et al., 1998), and assess the impact of compensatory growth in young deer which have been over-wintered on restricted and cost effective diets. Finishing units generally utilise high proportions of grain and compound supplements in winter diet and need cost-effective high energy (10.0–11.5 MJ ME/kg DM) roughages such as silage or hay as part of the balanced nutritional regime.

Intensive finishing units demand excellent management skills to counteract the negative impacts of behavioural stress inherent in very high stocking rates in newly weaned animals (Pollard and Littlejohn, 1997). Disease risks due to yersiniosis (Mackintosh, 1992*a*), parasitism (Waldrup et al., 1997) and trace mineral deficiency are all at a maximum during the autumn and early winter periods and all exaggerated by any nutritional deprivation and stress precursors like transport, relocation and cold wet weather conditions.

Effective management includes the provision of speciality pastures which are high in legume content and in annual grasses for maximum growth in autumn and early spring. Speciality crops like Puna Chicory (*Cichorium intybus*) are very high in feeding value and resistant to summer drought. Persistence of highly preferred species however is a problem under high grazing pressure (Li et al., 1997). Ideal grazing management also recommends that pasture swards are grazed precisely over winter/spring to leave a high residual DM content and pasture height of 10 cm surface height for maximum growth performance (Semiadi et al., 1992). Many finishing systems enhance efficiency of venison production through hybrid progeny from red deer hinds and wapiti type sires that can achieve optimal slaughter weights from 6–9 months of age (males) and 10–12 months (female). Biological efficiency of venison production has been modelled by Fennessy and Thompson (1998) demonstrating two slaughter peaks of efficiency at 30 and 68 weeks of age with a high proportional increase in biological efficiency due to hybridisation at the earlier age. This efficiency is conferred largely through lowering maternal costs with use of a smaller red hind as the dam, but feeding and management of the hybrid progeny requires the appro-

priate nutritional and health inputs. Such systems are entirely dependant on preserving reproductive rates by maintaining fertility and avoiding calf loss.

Sustainability of these operations are dependant on the economics of venison production, and the highly seasonal and variable return over the 10 month production season. Maintaining a high herd health status under imposed intensification requires good facilities, high standards of fencing and stockmanship to avoid death, injury and sub-optimal growth. Welfare concerns too are a developing issue with a clear conflict between a feedlotting and photoperiodic manipulated indoor wintering system and free choice shelter and supplementary feeding (Goddard and Matthews, 1998). To prevent land damage via soil compaction and fence tracking where large numbers of deer are confined at very high stocking rates some form of feedlotting or zero grazing has advantage through wet and cold conditions. This is viewed as feeding an appropriate diet at strategic times to enhance production as a substitute for pasture (Suttie et al., 1996*a*). Many developing farms in North America have little choice during long winter periods where practicalities require total supplementation. Wapiti and recently imported red deer adapt incredibly well to such conditions with the overwintering period improving subsequent ease of handling. The major problem is avoidance of the risk of 'grain overload' which is potentially fatal and created by abrupt changes in diet and a poorly managed introduction to new feeds. New Zealand farmers have also successfully adapted rotational grazing behind temporary electric fencing providing daily feed needs from saved grass in a carefully feed budgeted planned rotation around the farm. Increasingly the same feeding principles applicable to high producing dairy cows allow target weights and planned production to be a routine management skill.

That deer perform well in confined situations has been demonstrated for many years in the pen fed enclosures of China, although the primary focus is on velvet antler production.

Recent work by Gao (1996) contends that concentrates were traditionally oversupplied and that appropriate levels of crude protein in adult sika deer during antler growth are 19% and 19–23% for growing deer when considering the balance of antler production, weight gain, feed availability and feeding cost.

Future trends

The ecological sustainability of intensive deer farming systems present some conflict in terms of market perception of venison and an increasing consumer perception that food safety and appropriate welfare issues are integral in the presentation of the final product. Farm raised venison will be differentiated in the market place based on programmes that guarantee food quality through

total quality management starting at the farm (Pearse, 1993; NZGIB, 1995; CVC, 1996). With the economic security of excellent venison and velvet antler returns there are incentives to continue to improve ecological sustainability of deer farming through further stratification of stock class and production system with land form and capabilities. Cervids have already proven to be astonishingly adaptable in virtually every farming condition they have been exposed to, and improving knowledge of behaviour and the animal seasonality will continue to reinforce the need for ecological considerations to be a major factor in refining production systems.

Modern deer farming, irrespective of species of choice, tropical or temperate situation has a primary challenge as genetic improvement and selection procedures lead to the emergence of an on-farm strain of deer, for the specific farming environment, to develop appropriate feeding standards and systems to meet and exceed production demands. This in turn, for economic and welfare reasons requirements ongoing research and technology with deer is necessary in the areas of disease prevention, efficiency of reproductive performance and food safety. The management systems developed to date are an effective and encouraging portent for the future.

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GENETIC ANALYSIS OF FARMED DEER HYBRIDS

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Molecular methods which identify species-specific genetic markers are valuable for identification and genetic analysis of hybrid deer. We have used a variety of molecular techniques to compare Père David's deer *Elaphurus davidianus*, red deer *Cervus elaphus scotticus* and North American wapiti *C. e. manatobensis*. In total, these analyses have identified over 300 markers in which Père David's deer sires are distinct from farmed red deer and over 100 markers in which wapiti sires differ from red deer. Subsets of these markers have been used to identify hybrids on farms, and to estimate the proportion of red deer and wapiti in hybrid animals. Analysis of the segregation of the species-specific alleles in 352 backcross ¼ Père David's, ¾ red deer hybrids has produced a deer genetic linkage map which describes the chromosomal grouping and order of the markers. Correlation of the variability in phenotypic traits with marker segregation in the backcross hybrids has located genes which influence variation in gestation length, pedicle-initiation, birth weight, growth rate and a variety of morphological traits in Père David's deer × red deer hybrids. Such genetic analyses can now be extended, using new marker technologies, to more closely related taxa, such as farmed red deer and wapiti hybrids, and potentially analyse natural hybrid zones.

Key words: Farmed deer, hybridisation, genetic markers, *Cervus elaphus*, *Elaphurus davidianus*

The capture, translocation, and release of deer has been used for centuries to influence the genetics of natural populations and extend the range and diversity of game species. In many countries, this process has resulted in deer populations with mixed or hybrid ancestry (Lever, 1985).

While there are examples from other deer genera, hybridisation is possibly most widespread among members of genus *Cervus*. In Britain and Europe, red deer (*Cervus elaphus*) have been modified by translocation of animals between managed game preserves and hybridisation with taxa from other continents, including Asiatic and North American subspecies (wapiti) and sika deer (*C. nippon*) (Lorenz, 1953; Harrington, 1973; Bartos et al., 1981; Lever, 1985;

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Herzog, 1990; Abernethy, 1994). In Canada, farming of European red deer and red deer hybrids is perceived as a threat to natural populations of wapiti and is banned in some provinces (Lakusta, 1993). In China, the status of sika deer is a matter of debate, with some authors suggesting that Chinese sika are principally of hybrid origin (Lowe and Gardiner, 1975). In New Zealand, European settlers have introduced nine taxa to a variety of forest areas (King, 1990) and hybrid zones have developed between red deer (*Cervus elaphus scoticus*) and North American wapiti (*Cervus elaphus nelsoni*) (Nugent et al., 1987) and between red deer and sika (*Cervus nippon*) (Davidson, 1983).

During the 1970s large numbers of deer were captured from New Zealand's forests for farming. The crossing of red deer and wapiti subspecies and the production of novel hybrids, such as those between Père David's deer and red deer, have been a focus in strategies for the genetic improvement of these farmed deer (Fennessy et al., 1992; Pearse, 1992; Tate et al., 1992*a*). This paper reviews our research into the development of molecular genetic tools for the analysis of these farmed deer hybrids. The work has focused on two areas: firstly, the development of molecular tools to identify hybrid animals; and secondly, the use of interspecies hybrid pedigrees to locate specific genes which determine phenotypic differences between deer species and their hybrids.

Hybrid herds on New Zealand deer farms

Père David's deer × red deer: During 1984 and 1985 about 70 Père David's deer were introduced to New Zealand as a conservation measure and to investigate their utility for farming. Unfortunately over half of the imported animals succumbed to malignant catarrhal fever (MCF) within a few years of importation (Orr and Mackintosh, 1988) and deaths from the disease have restricted the herd to its current size of between 20 and 30 individuals. F_1 hybrids between red deer and Père David's deer were bred by artificial insemination to investigate the potential of altering the reproductive seasonality of farmed red deer. Père David's deer calve in early spring allowing calves to take advantage of the spring grass growth; in contrast red deer calve in late spring, too late to make full use of the spring pasture growth on New Zealand farms (Asher et al., 1988; Fennessy and Mackintosh, 1992).

The F_1 hybrids bred in New Zealand have produced over 400 calves in matings with red deer. The group most intensively recorded were calves born at AgResearch, Invermay Agricultural Centre, Mosgiel, New Zealand. These calves were conceived by natural mating and artificial insemination (AI) of red deer hinds (F_1 hybrid sires) or multiple-ovulation embryo transfer (MOET) using F_1 hybrid dams as donors (red deer sire). The breeding program, described in

detail by Tate et al. (1997), produced a total of 324 living backcross calves: 248 from AI, 48 from natural mating, 19 from embryo transfer and 9 calves born naturally to F_1 females.

The fertility of the F_1 Père David's deer × red deer hybrids is remarkable given the large genetic distance, Nei's (1972) D = 0.48 (Tate et al., 1992*a*), and the biological differences between the species. Père David's deer are distinct from red deer in seasonality (Loudon et al., 1989), behaviour (Bedford, 1951; Altmann and Scheel, 1980) morphology (Wemmer, 1983), mature size (Whitehead, 1993), and disease resistance (Orr and Mackintosh, 1988). In addition, Père David's deer differ from all other living deer in the structure of their antler (Whitehead, 1993). However, both species have the same number of chromosomes and very similar karyotypes (Wang, 1988).

Red deer × wapiti hybrids: Wapiti were introduced to the south-western fjords of New Zealand in 1905 (Donne, 1924). By this time, red deer populations (*C. e. scotticus*) introduced 30 years earlier were expanding rapidly and, by 1940, red deer and wapiti occupied the same range. Within ten years atypical animals, suspected to be hybrids were reported (Banwell, 1966). Subsequent studies of animals shot or captured from within the region suggest few, if any pure Wapiti remain (Caughley, 1971; Dratch, 1987*b*). Indeed, the rapid assimilation of these populations (which represent the extremes of body size and natural distribution of *Cervus elaphus*) provided evidence supporting the synonymisation of wapiti (previously *C. canadensis*) and red deer (*C. elaphus*) (Byrant and Maser, 1982).

On New Zealand deer farms, captured New Zealand wapiti, wapiti imported directly from Canada, and semen from North American wapiti have been used widely in breeding. Wapiti and wapiti-red deer hybrids are either farmed in their own right or used as terminal sires to produce rapidly growing calves from red deer. Imported wapiti and hybrids differ from red deer in mature size and growth rate and also in their greater susceptibility to internal parasitism, copper deficiency and increased resistance to malignant catarrhal fever (Mackintosh, 1992; Waldrup and Mackintosh, 1993). The body weight of adult wapiti stags on New Zealand farms ranges from 400 to 500 kg while red deer hinds are typically between about 95 and 115 kg. This size difference is such that production of first cross (F_1) animals requires specialist management, particularly at calving, to avoid dystocia, and during lactation to achieve the growth potential of hybrid calves. Typically animals perceived to be approximately ½–¾ wapiti are mated to red deer hinds while sires with a higher proportion of wapiti are mated to larger hinds (Pearse, 1992).

Identification of molecular markers

We have compared red deer (*C. e. scotticus*), wapiti (*C. e. manatobensis*) and Père David's deer using a variety of molecular genetic techniques: protein polymorphism, restriction fragment length polymorphism (RFLP), microsatellite analysis and amplified fragment length polymorphism (AFLP). The results of these screens for markers are summarised in Table 1. In each case the purpose of the screens was to identify differences between key parental animals (Père David's deer and wapiti sires) and red deer herds.

Table 1

Summary of genetic markers tested in Père David's deer and red deer

Marker type	No. markers	No. markers with species-specific alleles	
		Red vs. Wapiti	Red vs. Père David's deer
Protein polymorphism			
n ≥ 5 red, 5 wapiti, 5 PD	43	2	17
RFLP[1]			
n = 3 red, 3 wapiti, 3 PD	187	51	175
Microsatellites			
n ≥ 17 red, 17 wapiti, 5 PD	35	0	24
AFLP[2]			
n = 30 red, 2 wapiti, 2 PD			
(34 primer combinations)	> 1300	61	188

[1]RFLP = restriction fragment length polymorphism; [2]AFLP = amplified fragment length polymorphism; PD = Père David's deer

Protein polymorphism: Differences between red deer and wapiti have been identified in beta-globin (HBB), an unidentified plasma protein 'post-transferrin' (PTF) and vitamin D binding protein (GC) (Dratch, 1987*a*; Tate et al., 1988; Dratch et al., 1992; Stern et al., 1992). In addition, the subspecies show allele frequency differences in Cu-Zn superoxide dismutase (SOD1), transferrin (TF) and complement component (C3) (Dratch et al., 1992; Tate et al., 1992*b*; He et al., 1997). A study of over 40 protein loci (Tate et al., 1992*a*) found no new differences between red deer and wapiti. However, 19 loci showed fixed allelic differences between 5 Père David's deer and 5 red deer (Table 1). Further analysis of these proteins in a larger number of red deer (> 20) and Père David deer × red deer hybrids has supported the species-specificity of alleles.

Restriction fragment length variation: Restriction fragment length polymorphism (RFLP) analysis using single copy DNA probes (Southern, 1979) was

the first technique capable of scanning for DNA polymorphism on a genome-wide scale. We have applied RFLP analysis to the detection of species-specific markers in red deer, wapiti and Père David's deer. In total, 187 cDNA probes derived from either human, sheep, or cattle were hybridised to the DNA of three Canadian wapiti, three red deer, and three Père David's deer (Table 1 and Tate, 1997). At least 6 different restriction digests of these DNA samples were compared for each probe. The restriction enzymes which identified the most polymporhism were, in order, *Taq*I, *Msp*I, *Bgl*II, *Pvu*II *Pst*I, *Hin*dIII. The probes used are described in full by Tate (1997).

Of the 174 differences found between red deer and Père David's deer (Table 1), 162 were supported when a larger number of red deer and hybrids ($n > 50$) were genotyped. For the remaining 12 probes, the 'Père David's deer' allele occurred in red deer at a frequency of between 0.01 to 0.19. Only seven of the 51 red-wapiti differences in Table 1 have been analysed in a larger number of animals. In four cases the 'wapiti' allele was observed at low frequency (< 0.10) in red deer while the 'wapiti' allele was not observed in over 50 red deer analysed for the remaining three RFLP: a *Taq*I RFLP detected with bovine prolactin cDNA, a *Taq*I RFLP detected with a human fibronectin precursor cDNA (Kornblihtt et al., 1983), and a *Msp*I RFLP detected with the bovine luteinizing hormone receptor cDNA (Bacich et al., 1994).

Microsatellites: Microsatellite or simple tandem repeat markers have become a ubiquitous source of polymorphic markers for genetic analysis (Weber and May, 1989). These markers use the polymerase chain reaction (PCR) with specific primers to amplify DNA segments containing short simple repetitive DNA sequences, for example (AC)n. Approximately 20% of the thousands of microsatellite markers developed in sheep and cattle (Bishop et al., 1994; Crawford et al., 1995) also detect microsatellite variation in deer (Kuhn et al., 1996; A. Crawford, unpublished data).

A screen of 35 microsatellite markers in 17 red deer, 17 wapiti, and 5 Père David's deer identified 24 markers in which Père David's deer were monomorphic for an allele which did not occur in red deer (Table 1, Tate, 1997). No markers were identified with fixed allelic differences between red deer and wapiti but allele frequency differences were observed. Table 2 lists the allele frequency of three of these microsatellite markers, OarFCB5 (Buchanan et al., 1993), CelJP23 (Pemberton et al., 1995) and BtaRM188 (http://sol.marc.usda.gov/genome/cattle/) which were examined in a further 60 red deer and 150 Canadian wapiti. In each case the subspecies contained some common and some 'private' alleles (occurring in only one subspecies).

Amplified Fragment Length Polymorphisms (AFLP): Amplified fragment length polymorphism is a relatively new method, developed initially in plants (Vos et al., 1995). The method is designed to display and compare very large

numbers of DNA fragments based on sequence variation surrounding restriction enzyme sites. We have used the *Eco*RI, *Mse*I method described by Vos et al. (1995) to examine AFLP differences between red deer, wapiti and Père David's deer (Table 1). The deer were compared for 33 combinations of primers including 3, 4, or 5 selective nucleotides. The screen identified 61 fragments which were present in two wapiti but did not occur in 30 red deer and 188 bands which were present in two Père David's deer but did not occur in 30 red deer. These have yet to be analysed in a larger group of animals.

Table 2

Allele frequency of microsatellite markers, OarFCB5, CelJP23, and BtaRM188 in red deer and wapiti

Allele number	OarFCB5		CelJP23		BtaRM188	
	Red (n = 76)	Wapiti (n = 167)	Red (n = 64)	Wapiti (n = 176)	Red (n = 76)	Wapiti (n = 157)
1	0.171	0.003	0.008	0.573	0.007	–
2	0.105	0.003	0.008	0.022	0.125	0.013
3	0.191	0.003	0.195	–	0.105	–
4	0.171	–	0.063	–	0.513	0.061
5	0.033	–	0.008	0.113	0.059	0.010
6	0.276	0.090	0.117	0.285	–	0.006
7	0.007	–	0.211	0.008	0.072	0.092
8	0.026	0.003	0.352	–	0.020	0.815
9	0.007	–	0.008	–	0.059	0.003
10	0.007	–	0.031	–	0.039	–
11	0.007	0.108				
12	–	0.716				
13	–	0.039				
14	–	0.036				

Comparison of techniques: All four techniques identified differences between Père David's deer and red deer. The experimental procedures for microsatellite and AFLP techniques were least laborious. However, the AFLP technique had the advantage that a virtually unlimited number of primers and enzymes can be applied to identify new polymorphisms with minimal extra investment. RFLPs share this feature because of the large number of mammalian gene probes now available. In addition, RFLP markers are often associated with known genes for which functional and comparative genetic information may be available.

The methods based on restriction site polymorphism (RFLP and AFLP) appeared more effective for identification of species-specific markers than the

use of microsatellite markers. Microsatellite markers have a high mutation rate and mutations in different species may converge to produce similar genotypes (Bruford and Wayne, 1993). Convergent marker evolution (homoplasmy) is thought to be less likely for the gain of restriction sites, making them better markers for distinguishing closely related species (Dowling et al., 1996). While we have yet to evaluate the AFLP technique in a larger group of animals, our initial results suggest this could well become the method of choice for identification of specific genetic markers.

Application of species-specific markers

Hybrid identification: Even in farmed situations, hybridisation is often difficult to trace, either because animals have been captured from the wild, or because it has not been possible to record parentage accurately (Tate et al., 1990). Species-specific molecular markers provide an objective means to identify hybrid animals and, if a large number of species-specific markers are available, to estimate the proportion of genes from different species or subspecies in an animal.

The power of a 'hybrid test' is related to the number of independent markers and the degree to which these markers differentiate the two parental species. While the concept of absolute species-specificity is attractive, in most cases, it is impossible to prove. One solution is to assume that markers are never absolutely species-specific, but rather that alleles occur at very different frequencies in the two taxa. Where an allele is not observed its frequency is assumed to be some function of the number of reference animals examined (n) (eg: 0.5/n) (Dodds and Tate, 1997). This approach provides a robust basis for statistical analyses which estimate the level of admixture in an individual (Elston, 1971; McKlean and Workman, 1996; Dodds and Tate, 1997). We have used the statistical technique described by Dodds and Tate (1997) and subsets of the markers described above to estimate the proportion of red deer and wapiti (elk) in individual hybrids. Figure 1 gives an example of an estimate of the proportion of wapiti in a unknown wapiti × red deer hybrid. The estimate was based on data from 13 microsatellite markers. The error bars on the estimate could be reduced by analysis of a larger sample of markers.

Genetic analysis of hybrids: There is considerable world-wide effort to identify key genes which determine the productivity of farmed animals (Andersson et al., 1994; Haley, 1995). Hybrid populations provide a potentially valuable resource with which to conduct similar studies on deer because, in hybrids, the genes from separate species segregate producing measurable variability in phenotypic and farm production traits (Tate et al., 1997).

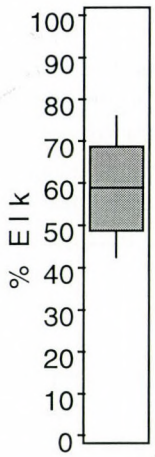

Fig. 1. Graphical depiction or 'Genometer' showing the proportion of wapiti in a hybrid between wapiti and red deer. The percentage and confidence intervals were estimated by the method outlined in Dodds and Tate (1997) using genotype data from 13 microsatellite markers including those presented in Table 3. The estimated percentage of wapiti (elk) in this hybrid is 59%; the 75% confidence interval for the estimate (box) is 49%–68% while the 95% confidence interval (whisker) is 43%–86%

We have examined the relationship between species-specific markers and trait variation in Père David's deer × red deer backcross pedigrees. This analysis required generation of a genetic linkage map describing the chromosomal location and order of species-specific markers in deer. Currently the map includes 16 protein polymorphisms, 148 restriction fragment length variants, 66 microsatellite markers (Tate et al., 1995; Broom et al., 1996; Montgomery et al., 1996; Tate, 1997) and 188 AFLP (M. L. Tate and K. M. McEwan, unpublished data). A subset of these mapped markers was then used to identify the chromosome segments inherited from red deer and Père David's deer in a group of 352 ¼ Père David's deer, ¾ red deer backcross progeny. Each of these hybrids was measured for a wide range of traits including, gestation length, birth weight and growth rate, seasonality, carcass quality, and morphological traits (Tate et al., 1997; Goosen, 1997). Analyses of this dataset have identified chromosome regions containing genes which influence gestation length, birth weight, weaning weight, growth rate, pedicle-initiation, and a range of morphological traits. Table 3 lists five examples of quantitative trait loci (QTL) detected in these analyses. Potentially, these markers could be used to assist in the selection of hybrids with desirable characteristics such as increased growth rate.

Table 3

Examples of quantitative trait loci (QTL) detected in Père David's deer backcross hybrids

Trait description	Location of QTL (deer linkage group)[1]	Size of effect[2]	% of variation explained[3]	Statistical significance (LOD)[4]	Reference
Gestation length of hybrid calf (red deer hind)	16	3 days	7.9	3.65**	Goosen et al., 1997
Birth weight of hybrid calf	4	640 g	6.0	3.04*	Goosen et al., 1998
Growth rate; weaning to 6 months	12	25 g/day	11.2	4.19**	Goosen, 1997
Live-weight at pedical-initiation	3	4.5 kg	10.0	3.06*	Goosen, 1997
Date of pedical-initiation	12	21 days	12.5	3.66**	Goosen, 1997

[1]The deer map contains 33 autosomal linkage groups; [2]The effect of substituting a red deer allele with a Père David's deer allele (absolute value); [3]'Size of effect' as a percentage of the total variance in the trait, within the backcross hybrid herd; [4]LOD = Log of the odds. The significance levels were estimated using simulations with a large number of iterations (n = 1000) and a normally distributed variable, *$P < 0.05$, **$P < 0.01$

Conclusions

New marker technologies offer the potential to identify a large number of species and subspecies-specific markers and conduct genetic analyses of hybrid deer. We have applied this technology to estimate the proportion of wapiti and red deer in crossbred animals and to locate chromosome regions influencing phenotypic traits in Père David's deer × red deer hybrids. The discovery of large numbers of wapiti-red deer differences using AFLP analysis also raises the potential of conducting similar genetic linkage studies in this subspecific cross. Related techniques could be used to analyse natural hybrid zones. However, in natural populations the use of larger numbers of animals to estimate the frequency of alleles in different taxa would be important since, unlike farmed deer, the exact parental animals could not be tested. Large numbers of species-specific markers could be used to estimate the level of hybridisation in individual animals and potentially to analyse the survival or phenotype of animals in natural hybrid zones.

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PHOTOPERIOD–MELATONIN RELAY IN DEER

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Long-lived mammals from cold and temperate climates, including many species of deer, express overt cycles in reproduction, moulting, fattening and other characteristics. These cycles persist under constant conditions, but are normally induced and entrained by the annual cycle in daylength. The photoperiod-relay involves the eyes, the suprachiasmatic nuclei (SCN) of the hypothalamus and the pineal gland which secretes melatonin only at night. The duration of daily melatonin secretion varies with daylength and provides an internal endocrine signal for the time-of-year. In deer, treatments with melatonin induce phase-shifts in all overt seasonal rhythms. Melatonin is thought to act on specific target cells in the brain and pituitary gland which express high affinity melatonin receptors. In sheep, micro-implants of melatonin placed in the mediobasal hypothalamus (MBH) induce a complete spectrum of short-day responses, while surgical disconnection of the pituitary gland blocks all photoperiodic responses except for the regulation of prolactin. These observations support the 'dual-site hypothesis' that melatonin acts primarily in the MBH to control gonadotrophin secretion and the reproductive axis, but acts primarily in the pituitary gland via the pars tuberalis, to control prolactin secretion and the pelage axis. This differential regulation helps explains how prolactin can be 'the hormone of summer' in all photoperiodic ungulates irrespective of their seasonal breeding characteristics.

Key words: Circannual rhythms, gonadotrophins, melatonin receptors, prolactin, seasonal breeding

Photoperiodic induction and entrainment

Since the pioneering studies by Jaczewski (1954) in male red deer (*Cervus elaphus*), and the comprehensive studies by Goss (1969*a, b*) in male sika deer (*Cervus nippon*), it has become well established that the annual cycle in photoperiod is the main environmental cue utilised by temperate ungulates to time seasonal rhythms in physiology and behaviour. In both red and sika deer, exposure to a change from long to short days induces premature reactivation of the reproductive axis. This involves an increase in gonadotrophin secretion and testicular activity, cleaning of the antlers and development of rutting behaviour, all features which normally occur in autumn. Conversely, a switch from short to long days is inhibitory to the reproductive axis (Goss, 1969*b*; Suttie et al., 1989).

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Regular changes between long and short days effectively accelerate the overt cyclicity such that as many as four reproductive cycles (and sets of antlers) can be produced within 12 months (Goss et al., 1974; Suttie et al., 1989). In addition, a phase-shift in the photoperiodic regimen causes a corresponding shift in the timing of the reproductive cycle (Goss, 1969*a*). These photoperiodic responses thus account for why deer readily readjust the timing of their breeding cycles when transferred across the Equator (Marshall, 1943; Goss, 1983).

While photoperiod has clear inductive effects on the seasonal reproductive cycle, deer do not need to experience changes in photoperiod to express long-term cyclicity. This was clearly demonstrated when sika deer stags were maintained under artificial photoperiods of constant long days or constant short days for several years (Goss, 1969*b*). Under these conditions the animals continued to cast and regrow the antlers periodically, reflecting the persistent long-term reproductive cycle, but the individuals were no longer well synchronised. The average period of the free-running antler cycle was found to be about 10 months (Goss, 1969*b*). More recent studies in female red deer have shown that long-term changes in prolactin secretion, voluntary food intake, and growth and moulting of the pelage also persist under prolonged constant long days (Loudon and Brinklow, 1992). The long-term cycles are thus generated endogenously as circannual rhythms, and under natural conditions the role of the changes in photoperiod is to induce and entrain the cycles. As a consequence the period of the endogenous rhythm is adjusted to match the sidereal year (e.g. 10 months expanded 12 months for sika deer), and the phase is set such that the timing of the physiological cycle occurs at the appropriate season (e.g. peak of the gonadal cycle in autumn rut).

In the deer species adapted to cold and temperate climates these intrinsic mechanisms of cycle generation and photoperiod-responsiveness ensure precise timing. In adult red deer, for example, it is not unusual for antler casting to occur within 3 days of the to same date in successive years for an individual, and for all animals of similar age to cast within 4 weeks (Lincoln, 1971; Lincoln et al., 1972; Fig. 1). This contrasts to the situation in sub-tropical species such as the axis deer (*Axis axis*) and hog deer (*Axis porcinus*) where individual males express clearly defined reproductive cycles (indicated by growth and casting of the antlers) with an approximate annual periodicity, but without marked synchrony amongst the males (Schaller, 1967; Mishra, 1982). This difference between species can persist even when animals are kept together at temperate latitudes (e.g. London zoo: Zuckerman, 1953; Loudon and Curlewis, 1988), which is consistent with the view that the basic cyclicity is endogenous. Furthermore, the failure of equatorial deer species to adjust to robust photoperiodic cues in temperate latitudes indicates that the photoperiodic mechanisms, which were probably evolved by the ancestral deer at higher latitudes, have become less responsive in equatorial species (Loudon and Brinklow, 1992).

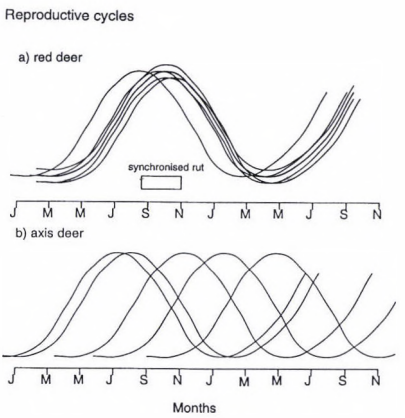

Fig. 1. Schematic diagram to illustrate the way the reproductive cycle in individual males is highly synchronised in male red deer (*Cervus elaphus*) but not in male axis deer (*Axis axis*). This applies in the natural habitat (red deer at 58 °N: Lincoln, 1971; axis deer at 0–30 °N: Schaller, 1967), and also when the species are kept together at temperate latitudes at the London zoo (Zuckerman, 1953; Loudon et al., 1989). Each solid line arbitrarily represents the testicular cycle in one adult individual

Pineal gland and melatonin

The full characterisation of the way the pineal gland, through its daily rhythm in secretion of melatonin, mediates the effects of photoperiod has been deduced largely from studies in rodents and the domesticated sheep (Turek and Campbell, 1979; Arendt, 1986; Reiter, 1991). The generalised scheme is summarised in Fig. 2. In mammals, unlike the other vertebrate groups, the pineal gland is not directly sensitive to light, and the photic information is transmitted from the lateral eyes. The neural relay involves non-visual, light-sensitive cells in the retina, the retino-hypothalamic pathway connecting to the suprachiasmatic nuclei (SCN) in the hypothalamus which act as the dominant central circadian rhythm generator (24-h clock), and a sympathetic innervation to the pineal gland. Destruction of any component in this neural relay interferes with the secretion of melatonin and renders animals unresponsive to changes in photoperiod. This applies to the removal of the eyes (Legan and Karsch, 1983), lesions of the SCN (Scott et al., 1995), sympathetic denervation of the pineal gland by superior cervical ganglionectomy (Lincoln, 1979), and removal of the pineal gland (Bittman et al., 1983).

The initial demonstration in sheep that disruption of melatonin secretion blocks the photoperiodic regulation of the seasonal characteristics was achieved by housing animals under an artificial lighting regimen designed to accelerate the natural period of the biological rhythm (e.g. use of alternating 16-week periods of long and short days to invoke a 32-week cycle; Lincoln, 1979). Under

these conditions, sheep without a functional pineal gland (pinealectomised or superior cervical ganglionectomised) were shown to be unable to adjust their long-term rhythms to the experimental lighting regimen. However, the animals still continued to express long-term changes in gonadotrophin and prolactin secretion, gonadal activity and other characteristics, presumably due to the intrinsic rhythmicity and/or the effects of other environmental cues which act independently of the pineal gland (Lincoln et al., 1989). A similar situation has been observed in pinealectomised white-tailed deer (*Odocoelius virginiana*) (Brown et al., 1978) and ganglionectomised in red deer (Lincoln, 1985) where the experimental surgery interferes with the precise timing of the annual cycles in reproduction, body weight and moulting, but does not block the expression of the seasonal cycles which can persist throughout life (Lincoln, 1992*a*). Congenitally blind deer without eyes can also express robust annual rhythms which remain remarkably well entrained to the natural environment (Jacobson and Waldhalm, 1992).

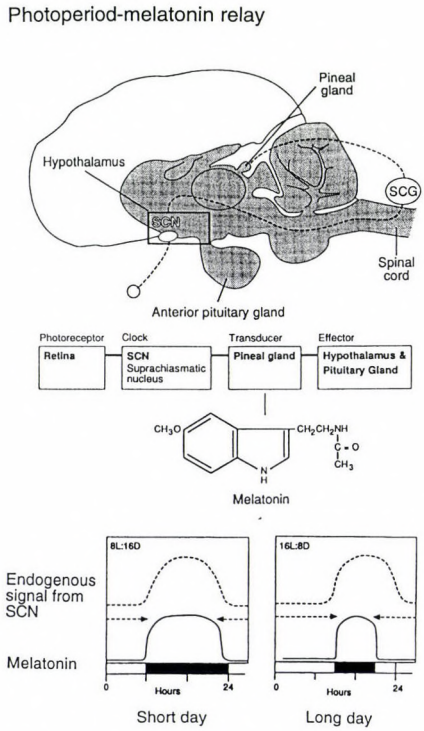

Fig. 2. Generalised plan of the photoperiod–melatonin relay. Light perceived by the lateral eyes acts: (*i*) to entrain the endogenous circadian rhythm generators in the SCN, and (*ii*) to inhibit melatonin secretion. Thus the light of the daily light/dark cycle dictates the duration of the nocturnal melatonin signal that encodes photoperiod. The melatonin signal acts via the hypothalamus and pituitary gland to time cycles in reproduction, moulting and other characteristics. Adapted from Lincoln (1992*c*)

The definitive evidence that the daily rhythm in melatonin secretion from the pineal gland mediates effects of photoperiod has come from studies in which pinealectomised ewes were treated with programmed infusions of melatonin. These were given to mimic the normal daily patterns in secretion of melatonin under long and short days (Bittman et al., 1983; Bittman and Karsch, 1984). Short-duration infusions (8-h daily) delivered for many weeks were found to suppress LH secretion, as normally occurs in pineal-intact animals exposed to long days, while long-duration daily infusions (16-h daily) had the reverse effect activating the reproductive axis as if under short days. The effect of the melatonin signal in pinealectomised ewes was unaffected by the ambient photoperiod (Yellon et al., 1985), or the phase of the light-dark cycle in which the treatment was applied (Wayne et al., 1988). The conclusion from these studies was that the *duration* of the melatonin signal is the critical parameter which conveys the effect of photoperiod: a short signal triggers the summer responses and a long signal triggers the winter responses. Under natural daylight outdoors, the daily duration of melatonin secretion varies in close parallel with the annual cycle in nightlength/daylength (Rollag et al., 1978; Arendt, 1985), thus there is a continuously changing internal signal which is used to govern the timing of the seasonal physiology.

The limited information available in deer supports the importance of daily melatonin signal in relaying the effects of photoperiod. Treatments with melatonin in spring and summer which effectively increase the duration of exposure to melatonin, induce short-day responses. This includes the premature reactivation of testicular activity and rutting behaviour in males (white-tailed deer: Bubenik, 1983; red deer: Lincoln et al., 1984; fallow deer: Asher et al., 1987), the early onset of ovulatory cycles in females (red deer: Webster and Barrell, 1985; Adam et al., 1986; fallow deer: Asher et al., 1988), and a shift in the cycle in appetite and growth of the winter pelage in either sex (red deer: Webster and Barrell, 1985; Milne et al., 1990). These effects can be produced by exogenous melatonin given daily in the afternoon to expand the daily melatonin phase, or by simply introducing a subcutaneous implant containing melatonin which permanently increases the circulating concentration of melatonin. Although subcutaneous implants produce a non-physiological, continuous melatonin signal, their effect when introduced under long days, is indistinguishable from that provoked by transfer to short days (sheep: O'Callaghan et al., 1989). Implantation of melatonin thus provides a convenient way to manipulate seasonality which has been commercially exploited in deer farming (Wilson et al., 1991; Asher et al., 1993).

Generation of the melatonin signal

Many studies in sheep have demonstrated that there is a close relationship between the daily pattern in the peripheral blood concentrations of melatonin

and the prevailing photoperiod. The levels of melatonin are invariably low during the day and at least 5-fold higher at night (Rollag et al., 1978; Arendt, 1985). The daily rhythm is regulated in part by the circadian rhythm generating system in the SCN, consequently the melatonin rhythm persists with a period close to 24 h in animals under constant darkness (Lincoln et al., 1985). The pattern of secretion under a normal daily light/dark cycle is also regulated by the inhibitory action of light on melatonin synthesis and secretion. This inhibitory effect is readily demonstrated by exposing animals to light at night; in sheep light intensity as low as 1 lux is effective at suppressing the release of melatonin (Ravault and Thimonier, 1988; Arendt and Ravault, 1988). Experimental studies indicate that the entrainment effect of light sets the phase of the melatonin rhythm, but the *inhibitory* effect of light determines the variation in the duration of melatonin secretion between long and short days (Picazo and Lincoln, 1995). This effect is clearly evident under long days where the long light phase 'masks' the expression of the underlying melatonin rhythm to produce the characteristic short-duration melatonin signal of summer (Fig. 2).

Daily rhythms in the blood concentrations of melatonin, with increased levels at night and low levels during the day have been described in white-tailed deer (Bubenik and Smith, 1987) and reindeer (Eloranta et al., 1992; Stokkan et al., 1994). The situation in the reindeer is of particular interest since the animals used in this study were living at high latitude (approximately 70 °N) with constant light in summer and constant darkness in winter. Under these conditions the diurnal rhythm in the blood concentrations melatonin was expressed throughout spring (February–April) and throughout autumn (August–November), closely reflecting the rapidly changing photoperiod over the equinoxes. The diurnal rhythm was absent, however, during the constant light of summer and constant darkness of winter (Stokkan et al., 1994). In summer, this lack of rhythmicity is readily explained since the light intensity even at midnight is likely to be suppressive to melatonin secretion. Indeed, transfer of reindeer indoors into darkness in summer even at midday results in an immediate increase in the blood concentrations of melatonin (Eloranta et al., 1992). In winter, the lack of rhythmicity may indicate that a periodic light signal is required to maintain the rhythm in melatonin secretion. This is consistent with the observation in sheep that the introduction of a 1-h light pulse every 24 h re-induces a robust diurnal rhythm in melatonin secretion in animals exposed to prolonged constant darkness (Ebling et al., 1988).

Melatonin receptors in the brain and pituitary gland

The potential sites in the brain and pituitary gland where melatonin acts to regulate seasonal physiology have been investigated by mapping the distribution of binding of iodomelatonin using *in situ* autoradiography (sheep: Bittman and

Weaver, 1990; goat: Daveson et al., 1992; red deer: Williams et al., 1996; Fig. 3). The specific binding is taken to represent the presence of membrane-bound receptors and thus the location of the target cells that respond to melatonin. Binding of iodomelatonin occurs in multiple sites in the brain, particularly in the hippocampus and septum, but also in the cerebellum, cortex, putamen, superior colliculus and choroid plexus. The highest concentration of binding sites, however, invariably occurs in the pars tuberalis of the pituitary gland. The binding sites have been shown to be time-dependent and saturable with a binding constant in the low picomolar range consistent with a high affinity receptor (Williams et al., 1996). In addition, the binding in all areas except the hippocampus was inhibited in the presence of the synthetic nucleotide, GTPgS, indicative of a G-protein coupled receptor.

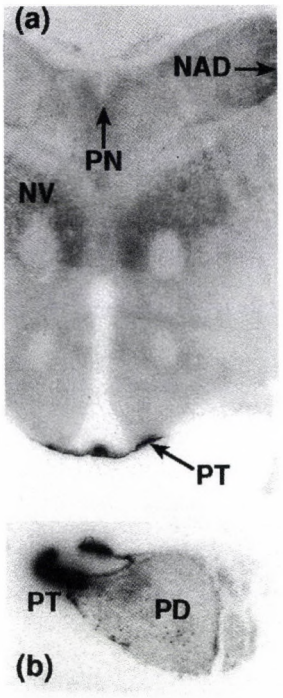

Fig. 3. In situ autoradiograph of a coronal section of: (a) brain and (b) pituitary gland in the red deer (*Cervus elaphus*) showing total [2-125I]iodomelatonin binding and the potential target sites for the action of melatonin. Abbreviations: NV, nucleus ventralis; PT, pars tuberalis; PD, pars distalis; PN, paratenialis nucleus. Adapted from Williams et al. (1997)

Two melatonin receptor genes, designated Mel 1a and Mel 1b, have been fully characterised (Reppert et al., 1994; Reppert and Weaver, 1995). The two receptor proteins have about 60% homology and both show high affinity binding for melatonin. The exon/intron organisation of the Mel 1a receptor and generalised structure of the Mel 1a receptor protein is illustrated in Fig. 4. The Mel 1b receptor is expressed largely in the retina and is thought to regulate retinal physiology, while the Mel 1a receptor is expressed in the pars tuberalis, SCN and other neural sites, and probably mediates the multiple effects of melatonin on seasonality (Reppert and Weaver, 1995). This fits with the finding that a mutation of the Mel 1b receptor gene which renders it functionless has no effect on seasonal and circadian responses to melatonin in the Siberian hamster (*Phodopus sungorus*) (Weaver et al., 1996). In sheep, Mel 1a receptor mRNA is present in high concentrations in the pars tuberalis as revealed by *in situ* hybridisation (Reppert et al., 1994; Williams et al., 1997), however, the localisation of Mel 1a receptor mRNA in specific sites in the brain has so far not been reported possibly due to the low level of expression.

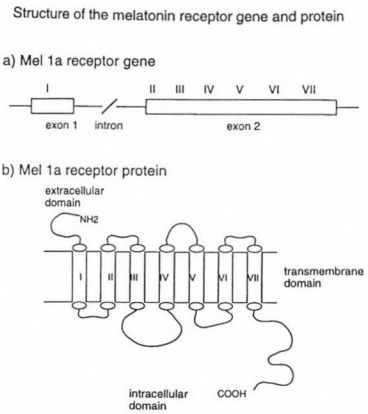

Fig. 4. Structural organisation of the Mel 1a receptor gene and the Mel 1a receptor protein. The protein is expressed in the pars tuberalis, SCN and other sites in the brain, binds melatonin with high affinity and most probably relays the effects of melatonin on seasonal cyclicity. Adapted from Reppert et al. (1994) and Reppert and Weaver (1995)

Sites of action of melatonin in control of seasonality

Melatonin has been administered locally in different regions of the hypothalamus and pituitary gland to elucidate where the hormone acts to control the seasonal responses in sheep (Lincoln and Maeda, 1992*a,b*; Malpaux et al., 1993). This has involved placing bilateral micro-implants containing melatonin in possible target sites in animals preconditioned under long days. The prediction was that any treatment at a site where the endogenous (short-duration)

melatonin signal is acting would block the effect of long days. In Soay rams, treatments with melatonin in the mediobasal hypothalamus (MBH), and to a lesser extent in the adjacent pars tuberalis, but not in other sites (POA, septum, pars distalis), were consistently effective in blocking the effect of long days (Fig. 5). This included the activation of gonadotrophin secretion and growth of the testes (Lincoln and Maeda, 1992*a*; Lincoln, 1994), induction of β-endorphin secretion and associated changes in body weight, and suppression of prolactin secretion and growth of the winter coat (Lincoln and Maeda, 1992*b*). The delivery of a more physiological intermittent, daily infusion of melatonin into the MBH using dialysis probes was also effective at provoking short-day responses (Lincoln, 1992*b*).

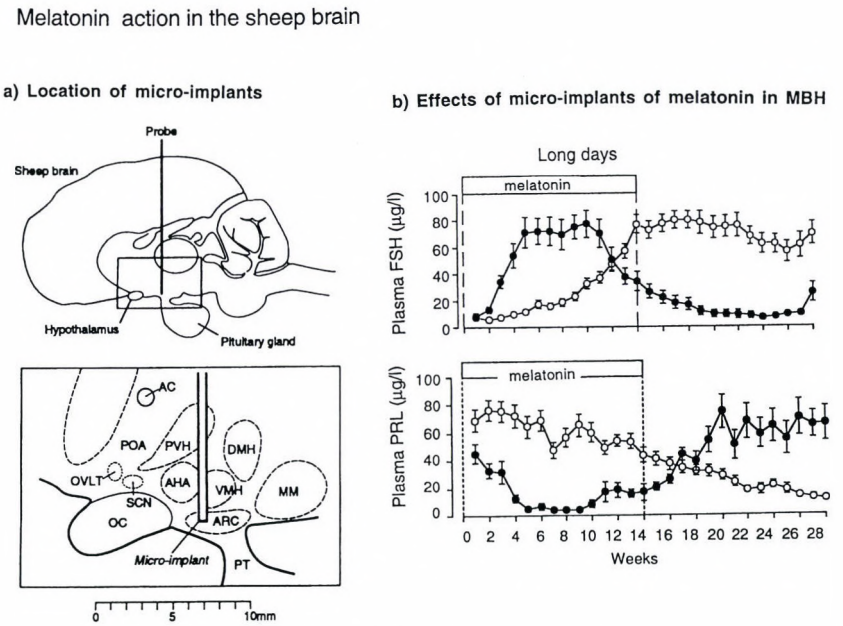

Fig. 5. (a) Localisation of micro-implants of melatonin in the mediobasal hypothalamus (MBH) in Soay rams. The implants consisted of stainless steel needles with melatonin fused inside the tip. They were placed bilaterally in the brain and left in place for 14 weeks while the animals remained under long days. (b) Changes in the blood plasma concentrations of FSH (above) and prolactin (below) in a group of Soay rams treated with melatonin implants in the MBH as above (•), or treated with empty implants or no surgery as a control (o). Only treatments in the MBH produced a full spectrum of short-day responses. Key abbreviations: AHA, anterior hypothalamus; ARC, arcuate nucleus; PT, pars tuberalis; OC optic chiasm; SCN, suprachiasmatic nucleus; VMH, ventromedial hypothalamus. Adapted from Lincoln and Maeda (1992*a*,*b*)

Similar results have been obtained in ewes, where melatonin delivered continuously within or close to the MBH, but not in the anterior and lateral hypothalamus, was effective at reactivating LH secretion in oestrogen-implanted

ovariectomised animals housed under long days (Malpaux et al., 1993). Treatments in the region of the pars tuberalis, however, were only effective in altering prolactin secretion (Malpaux et al., 1995). This has been used as evidence that the cells in the pars tuberalis, which express the very high concentration of melatonin receptors, do not mediate photoperiodic effects on gonadotrophin secretion and the reproductive axis, although they may relay the effects on prolactin secretion and its dependent functions.

This concept has been investigated in detail using Soay rams in which the pituitary gland has been surgically separated from the hypothalamus (hypothalamo-pituitary disconnected, HPD). In HPD rams, the blood concentrations of prolactin were found to change in response to photoperiod and melatonin administration in a similar manner to control animals, and the moult cycle also persisted (Lincoln and Clarke, 1994). This was despite evidence that the central regulation of prolactin had been removed, and that dopamine was no longer controlling prolactin secretion (Lincoln and Clarke, 1995). In these animals, the circulating levels of gonadotrophins were permanently very low due to the presumed absence of gonadotrophin releasing hormone (GnRH) from the hypothalamus. The testes were also markedly regressed but showed some residual cyclicity related to the changes in prolactin (Lincoln et al., 1996). Overall, these results provide the best evidence so far available that melatonin acts directly in the pituitary gland to modulate prolactin secretion. Since melatonin receptors are not expressed by lactotrophs in sheep (Williams et al., 1997), this effect may be relayed via melatonin-responsive cells in the pars tuberalis. This is supported by the recent observation that pars tuberalis cells secrete a factor called 'tuberalin' which stimulates prolactin secretion, and which is thought to be under inhibitory regulation by melatonin (Morgan et al., 1996; Hazelrigg et al., 1996).

The HPD sheep model has also been used to assess the impact of melatonin on the secretion LH and FSH. This involved the chronic administration of a pulsatile infusion of GnRH to reactivate the reproductive axis in HPD Soay rams living under long or short days (Lincoln and Clarke, 1998). This treatment induced an increase in gonadotrophin secretion and full maturation of the testes, but there was no effect of the prevailing photoperiod on these responses. Thus it is unlikely that the melatonin signal acts directly in the pituitary gland to regulate the seasonal reproductive cycle.

The more probable site of action for the control of the reproductive axis is in the MBH. This is supported by the micro-implant studies where melatonin placed in the MBH reactivated pulsatile LH secretion in hypothalamic-intact sheep, a response dependant on the release of GnRH from the hypothalamus. It is well established that seasonal changes in gonadotrophin secretion are induced by an increase in the pulsatile release of GnRH which via the pituitary portal vascular activates the gonadotrophs (Rhim et al., 1993). This favours the view that melatonin modulates the release of GnRH, possibly by acting on the dopa-

minergic and opioidergic neural networks in the MBH which synapse with the GnRH neurones (Rasmussen, 1991). In the Syrian hamster (*Mesocricetus auratus*) lesions in the ventromedial region of the MBH, an area with iodomelatonin binding sites, blocks the action of melatonin on gonadotrophin secretion, but notably spares the prolactin response (Maywood and Hastings, 1995). This is clear evidence that melatonin has more than one site of action in the control of seasonal rhythms.

Dual-site hypothesis

The working hypothesis based on the current evidence is that the melatonin signal that encodes photoperiod acts primarily in the pituitary gland to mediate effects of photoperiod on prolactin secretion and its dependant responses [e.g. pelage cycle (Lincoln, 1989)], but acts primarily in the MBH to mediate effects of photoperiod on gonadotrophin secretion and its dependant responses (e.g. gonadal cycle). The concept is summarised in Fig. 6. How the melatonin signal interacts to affect the circannual rhythm generating systems is still a mystery, but this may occur separately at the level of the pituitary gland for prolactin and at the level of the hypothalamus for gonadotrophins. The differential regulation of these two endocrine systems provides an explanation for the apparent *paradox* that all photoperiodic mammals are essentially similar with regard to prolactin secretion with a maximum in summer associated with the growth of the summer pelage. But there is much species-variation in the timing of gonadotrophin secretion and the mating seasons (spectrum from long- to short-day breeders). The lack of a fixed temporal relationship between gonadotrophin and prolactin secretion is well illustrated amongst the different species of deer. Prolactin annual profiles are similar in white-tailed deer (Mirarchi et al., 1978), roe deer (Schams and Barth, 1982), reindeer (Lincoln and Tyler, 1994), rusa deer, *Cervus timorensis* (Van Mourik and Stelmasiak, 1993), Eld's deer, *Cervus eldi thamin* (Montford et al., 1993), fallow deer (Asher et al., 1993) and red deer (Loudon et al., 1989), while in Pere David's deer the annual cycle peaks some 6 weeks earlier associated with an earlier development of the summer coat (Loudon et al., 1989). The seasonal maximum in gonadotrophin secretion and the rutting season, however, occurs in spring (rusa and Eld's), summer (roe and Pere David's), autumn (red and fallow) and early winter (white-tailed and reindeer) unrelated to the prolactin cycle (Lincoln, 1985; Montford et al., 1993).

Overall, it appears that the pituitary mechanism for the photoperiodic regulation of prolactin has been highly conserved across species, and prolactin remains the 'hormone of summer'. In contrast, the hypothalamic mechanisms for the photoperiodic regulation of gonadotrophins have been modified by the selection to achieve the optimal time for conception which varies between species related to body size/gestational length and the seasonality of the natural environment.

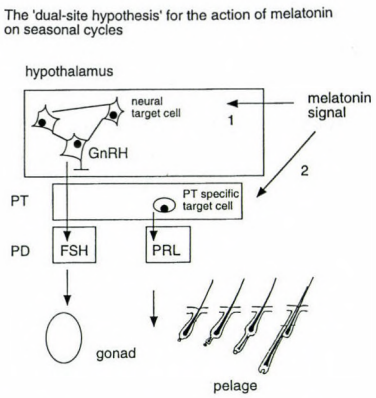

Fig. 6. Summary of the 'dual-site hypothesis' which proposes that the melatonin signal acts: (1) primarily in the mediobasal hypothalamus via neural target cells to mediate effects of photoperiod on the gonadotrophin/reproductive axis, and (2) primarily in the pituitary gland via PT specific cells to mediate effects of photoperiod on the prolactin/pelage axis. Abbreviations: FSH, follicle stimulating hormone: GnRH, gonadotrophin releasing hormone: PD, pars distalis: PT, pars tuberalis; PRL, prolactin

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THE INFLUENCE OF SEXUAL DIMORPHISM IN BODY SIZE AND MOUTH MORPHOLOGY ON DIET SELECTION AND SEXUAL SEGREGATION IN CERVIDS

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In mammals patterns of food resource distribution influence female distribution, leading to aggregation and favouring the evolution of a polygynous mating system. Under polygyny, sexual selection favours an increase of the male body size, since larger bodied males have competitive advantage in fights for mates. As a result, sexual body size dimorphism is a general rule in polygynous artiodactyls and is correlated with the degree of polygyny. Sex differences in body size lead to differences in energy requirements and food selection between the sexes. This has led to the sexual size dimorphism hypothesis being used to explain sexual segregation in ungulates, although from the available studies, it is not possible to deduce a consistent pattern between sexes in the use of forage of different abundance or quality. Two other groups of hypotheses have been put forward to explain sexual segregation in ungulates. These are based on reproductive strategy and social factors, both of which are independent of body size. The mechanistic explanation for differences in food selection ability and intake rate between animals of different body size and how this can lead to an understanding of the sex differences in diet and sexual segregation, both of which are intimately linked, is discussed.

Key words: Allometry, cervids, diet selection, sexual dimorphism, sexual segregation

Measuring segregation: social, habitat or spatial segregation between sexes?

In the literature sexual segregation has commonly been measured using the classical ecological overlap and association indexes (i.e. Schoener's, Dixon's or Dice's), however the use of these indexes can lead to confounding results because they are stochastically related to sex ratio, population density and group size (Conradt, 1998). An additional problem is that the definition of sexual segregation is confusing in the terms in which it is based. Sexual segregation can occur in different ways depending on the 'aggregation unit' one considers, that

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is, the 'aggregation unit' can be (*i*) the social group, in this case a definition of group should be established, (*ii*) the habitat type, or (*iii*) simply the space occupied (i.e. grid squares on a map). To avoid inconsistent comparisons between indexes from different studies, Conradt (1998) has proposed a segregation coefficient which is free of stochastic relations and can be applied to distinguish social, habitat or spatial segregation of the sexes. The coefficient takes values between 0 in the case of no segregation and 1 in the case of complete segregation. This is a major step forward in the study of sexual segregation and will help to standardise research in the future.

Are the sexual diet differences and sexual segregation two measurements of the same issue?

A number of hypotheses have been proposed to explain sexual segregation in polygynous ungulates (Fig. 1). They can be summarised into three groups (Main et al., 1996), although they are not mutually exclusive, namely (*i*) *reproductive-strategy* — female and male behaviour differs due to different selection pressures to optimise reproductive success; females avoiding risks to neonates at the expense of nutrient intake (Main and Coblentz, 1996), whereas males accumulating reserves to invest in increasing body size to ensure competitive success in the rut (Clutton-Brock et al., 1982; Hedrick and Temeles, 1989; Prins, 1989), (*ii*) *sexual dimorphism-body size hypothesis* — allometric differences between body size and bite size lead to different intake abilities between the sexes (Clutton-Brock and Harvey, 1983; Illius and Gordon, 1987). This hypothesis is not independent of the reproductive-strategy hypothesis since sexual dimorphism in body size could be the mere consequence of the reproductive strategy, and (*iii*) *social-factors hypothesis* — males and females differ in their ontogenetic behaviour, showing different levels of activity and patterns of interaction leading to a social auto-segregation by sex-age classes (Bon and Campan, 1996).

The reproductive-strategy and the social-factors hypothesis can explain differences in the habitat use between sexes, independent of differences in their energetic requirements. The sexual dimorphism-body size hypothesis is, however, intimately related to the energetic requirements and differences in foraging optimisation between the sexes (Fig. 1). Diet selection has been widely used to explain habitat use differences between species. However, the sexual dimorphism–body size hypothesis has received much criticism as an explanation of the sexual segregation in artiodactyls in the wild (Weckerly and Nelson, 1990; Miquelle et al., 1992; Weckerly, 1993; Jenks et al., 1994; Gross et al., 1995*a*; Main and Coblentz, 1996), although there have been no experimental tests of this hypothesis in polygynous artiodactyls.

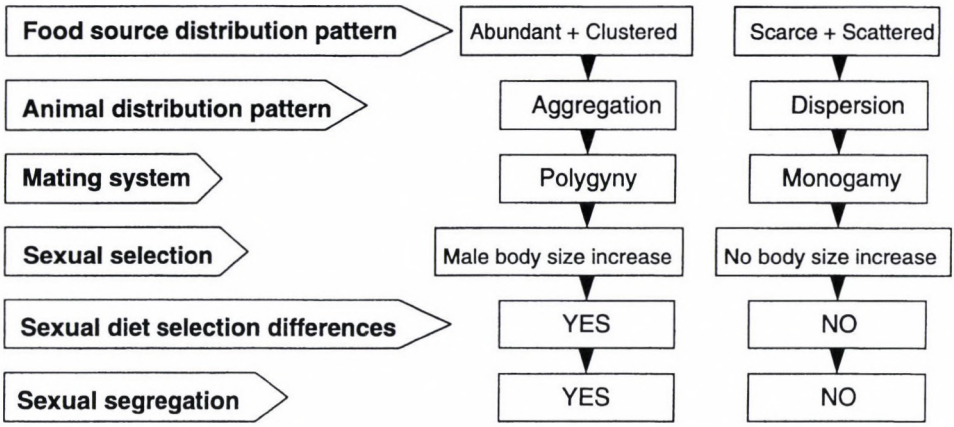

Fig. 1. The conceptual relationships between sexual body size dimorphism, diet selection differences between sexes and sexual segregation in artiodactyls. The origin of sexual dimorphism seems to be due to differences in the food source distribution pattern. Abundant and clustered food sources enable animals to exist at high population densities resulting in high degree of aggregation. In this environment, a polygynous mating system is favoured and sexual selection for larger-bodied males, which are better competitors in conflicts for mates. Body size differences between sexes lead to differences in energetic requirements and, therefore, differences in diet selection, which, as in last resort, results in sexual segregation

The use of different diets by either sex can be explained using the sexual dimorphism–body size hypothesis on the basis of three concepts, namely (*i*) males are less competitive feeders on short swards than are females due to allometric differences in incisor breadth ($W^{0.33}$, Clutton-Brock and Harvey, 1983; Illius and Gordon, 1987), which scales with body weight below the allometric relationship for maintenance energy ($W^{0.75}$), (*ii*) larger-bodied males are less selective feeders because of their greater daily requirements for food (Beier and McCullough, 1990; Staines and Crisp, 1978), and (*iii*) males select habitats with abundant, low-quality forage because their larger rumino-reticular volume makes them more efficient at converting fibre into energy, allowing them to subsist on lower quality forage (Demment, 1982; Demment and Van Soest, 1985).

The sexual dimorphism–body size hypothesis states that small body-sized animals outcompete larger ones when they graze together on short swards (Gwynne and Bell, 1968; Bell, 1971; Illius and Gordon, 1987). Under this hypothesis it is expected that females use short but highly nutritious swards, while males use more abundant but poorer quality pastures (Clutton-Brock et al., 1982). There are other factors which indirectly correlate dietary differences with body size independently of the relationship between body size and energy requirements. For example, predation risk involves both sexes in different ways, males, being larger, are not as vulnerable to predation as are smaller body-sized

females (Bleich et al., 1997). In these conditions males are less restricted in their habitat use and can access a wider range of food resources than can females. Here one would predict that in dimorphic species the males would occupy high quality habitats with high predation risk, whereas these habitats would be avoided by the females (Main and Coblentz, 1996; Bleich et al., 1997). However, this hypothesis by itself cannot explain dietary differences and sexual segregation in habitats free of predators, although there may be legacy from the past. The common problem that dominates the sexual segregation literature is that most conclusions are based on observational studies, where several factors are interacting and it is difficult, if not impossible, to understand the real contribution of each factor.

In order to generalise the sexual dimorphism–body size hypothesis to the Cervidae it should be necessary to demonstrate the existence of (*i*) body size dimorphism, and/or (*ii*) allometric bite size differences between sexes, and/or (*iii*) differences in digestive capacity between sexes, which lead to differences in habitat or vegetation community use. It should be noted that sexual segregation would occur at the level of vegetation use only if the quality and/or quantity of forage is patchily distributed at the scale of the vegetation community enabling aggregation of individuals of the same sex. If quantity and quality variation is at the level of within communities, a very fine mosaic of food resources would enable the sexes to achieve different diets, without necessity of sexual spatial segregation.

Forage-abundance or nutrient forage quality hypotheses: is there a defined pattern between the sexes?

Studies on dietary differences between the sexes in cervids, conducted in the same area and at the same time, which provide reliable comparative year-round information, have occurred in a few species only (white-tailed deer, *Odocoileus virginianus*: Beier, 1987; Weckerly and Nelson, 1990; Jenks et al., 1994; black-tailed deer, *Odocoileus hemionus*: Bowyer, 1984; Ordway and Krausman, 1986; Scarbrough and Krausman, 1988; Weckerly, 1993, 1994; Main and Coblentz, 1996; red deer, *Cervus elaphus*: Watson and Staines, 1978; Clutton-Brock et al., 1982; Staines et al., 1982; Groot-Bruinderink and Hazebroek, 1995; moose, *Alces alces*: Miquelle et al., 1992; Miller and Litvaitis, 1992; fallow deer, *Dama dama*: Thirgood, 1996). They have detected higher diet quality for males (black-tailed deer, Main and Coblentz, 1996); higher diet quality for females (red deer, Staines et al., 1982; white-tailed deer, Beier, 1987); males using habitats with more abundant forage (red deer, Clutton-Brock et al., 1982; moose, Miquelle et al., 1992); no differences between sexes in the quality of the diet or

inconsistent variation between years (red deer, Groot-Bruinderink and Hazebroek, 1995; white-tailed deer, Weckerly and Nelson, 1990).

A common confounding issue in most studies is the assumption that diet quality is positively related to habitat forage quality. Frequently, habitat forage quality is deduced from the vegetation community used, without chemical analyses being conducted to estimate the real nutrient quality of the diet. Furthermore, habitat use is deduced from direct or radio-tracking records which do not always separate out grazing activity from other behaviours (Watson and Staines, 1978; Bowyer, 1984; Ordway and Krausman, 1986; Scarbrough and Krausman, 1988; Miller and Litvaitis, 1992; Weckerly, 1994; Thirgood, 1996). Observations of what vegetation is being consumed, and estimation of feeding activity, should be made to determine the intensity of vegetation use (e.g. bite size and bite rate) if a real estimate of the nutritional value of habitat use is to be determined. Ideally, this would lead to estimates of the instantaneous intake rate of digestible nutrients. In the wild, direct observation of feeding bouts can give enough accuracy to define the forage that is being used (Weckerly, 1994), but in communities comprising very small, evenly distributed patches, estimation is more difficult, if not impossible. Estimates can be achieved by analysis of rumen content or faeces (Staines et al., 1982; Beier, 1987; Miquelle et al., 1992; Jenks et al., 1994; Groot-Bruinderink and Hazebroek, 1995; Main and Coblentz, 1996).

Two hypotheses are commonly tested in relation to the response to forage abundance (i.e. forage-abundance hypothesis) or nutrient quality of forage (i.e. selective-quality hypothesis, Weckerly, 1994) between sexes. From the reported results about the diet of both sexes is not possible to deduce a consistent pattern. Some studies have detected that females in summer attempt to maintain maximum volume of digesta in their gastrointestinal tracts in comparison with males, while during winter selective-quality hypothesis seems to mark the differences between sexes (Jenks et al., 1994). Other studies point out that the habitat use, and, therefore, diet selection, is influenced by the greater requirements for water of lactating females, resulting in the use of habitats closer to water sources (in black-tailed deer, Bowyer, 1984).

Allometric differences between sexes

Sexual body size dimorphism

Body size or morphological sexual dimorphism can confer foraging advantages to either sex in different habitats. Only recently has the existence of body size sexual dimorphism in artiodactyls been demonstrated when the phylogenetic effect has been removed (Loison et al., in press; F. J. Pérez-Barbería, I. J. Gordon and C. M. Janis, unpublished data). The evolutionary cause of sexual

body size dimorphism in cervids is uncertain, although most studies point out that differences are probably due to sexual selection in favour to larger males (Fig. 1). The degree of sexual body size dimorphism has been associated with the level of polygyny (Cheverud et al., 1985; Clutton-Brock, 1985, 1991*a*; Loison et al., in press). Loison et al. (in press) found that the relationship between sexual dimorphism and polygyny level occurs because of the positive correlation between polygyny level and body mass. They pointed out that this is especially clear for ruminants, where very small species are not polygynous and the larger ones are not monogamous. The association between body size and mating system (monogamy – polygyny) seems to be linked to ecological variables that constrain the use of highly energetic but scarce food resources (Jarman, 1974; Clutton-Brock and Harvey, 1978; Fig. 1). The scattered spatial distribution of these resources limits population density and aggregation of the females of species which in turn would determine the male mating system that is optimum in terms of maximising reproductive success.

Sexual morphological dimorphism

The hypothesis that predicts differences in the muzzle, mouth and teeth shape is based on females having higher energetic requirements per unit body weight (i.e. pregnancy and lactation) during a significant period of their life history in comparison with males (Kay, 1978; Shine, 1989). For example, it has been found in white-tailed deer that lactating females have, in absolute terms, a longer gastrointestinal tract and more digesta within the rumen and intestines than do males in summer and winter (Jenks et al., 1994). Whether females actually do have higher metabolic requirements during their life history has been widely discussed (see Clutton-Brock, 1991*b*, for a wide range of examples and discussion).

The evidence for sexual morphological dimorphism of some parameters involved in intake rate (i.e. incisor breadth), food selection ability (i.e. muzzle width, incisor protrusion) and food comminution effectiveness (i.e. post-canine occlusal surface area) appears to be lacking. For example, after removing the effects of body size and phylogeny, no sex differences were found in any of the mouth parameters for artiodactyls (F. J. Pérez-Barbería, I. J. Gordon and C. M. Janis, unpublished data). However, body size sexual dimorphism could be enough to produce differences in diet composition between the sexes, because smaller body size confers greater ability to be selective while feeding.

Polygynous ungulate females, due to being smaller than males (Silva and Downing, 1995; Loison et al., in press; F. J. Pérez-Barbería, I. J. Gordon and C. M. Janis, unpublished data), have narrower muzzles which enable them to access smaller discrete high energy items of food from intimate mixtures of herbage (Pérez-Barbería and Gordon, unpublished data).

Higher intake rates for the smaller sex?

Body size differences between the sexes can confer some physical advantages to the masticatory apparatus of the smaller sex. If we assume a relationship between chewing frequency and bite rate, both being rhythmic movements, bite rate could be conditioned by the metabolic rate, as Fortelius (1985) suggested for chewing frequency. Fortelius (1985) found that chewing frequency scaled to body mass (M) to the –0.25 power in 9 species of herbivore mammals, and he argued that it scaled to metabolic rate ($M^{3/4}$) if one assumes that the volume comminuted per chew is isometric to body mass M^1 and the food comminuted per unit time scales as $M^1 \times M^{-1/4}$, that is, to $M^{3/4}$. However this hypothesis has some serious objections, one of which is that the assumption that the bite size scales to M^1 does not appear to be correct. Shipley et al. (1994) found that the maximum bite size scaled to $M^{0.72}$ in 12 species of herbivore mammals. Moreover, more precise estimations of the relationship between chewing frequency and body mass indicate a significantly shallower scaling relationship than that predicted by Fortelius (1985), i.e. $M^{-0.129}$ and $M^{-0.19}$ calculated for 26 and 12 species of herbivore mammals by Druzinsky (1993) and Shipley et al. (1994) respectively, reducing credence for the metabolic hypothesis of Fortelius (1985).

A more realistic hypothesis to explain the scaling exponent of chewing frequency is based on physical constraints of the components of the masticatory apparatus. Druzinsky (1993) used the Turvey et al. (1988) model, which is based on a hybrid mass-spring/simple pendulum, under the prediction that the period of chewing (i.e the reciprocal of chewing frequency) should scale with jaw length to the 0.5 power. Druzinsky (1993) found a power of 0.42, which was not significantly different from 0.5 power. This suggests that animals with shorter jaws can chew faster than those with longer jaws. Shipley et al. (1994) discussed some of the selection pressures against the development of shorter jaws as an adaptation to different type of diets. Nevertheless, the physical advantages of faster chewing in animals with shorter jaws have been demonstrated (Druzinsky, 1993).

We should expect a similar reasoning for bite rate, although with the added confounding effect of the influence of the physical form of the food. This increases the variability of the bite rate in comparison with the chewing frequency variability during mastication, because the food processing time is higher during cropping than during comminution (Pérez-Barbería and Gordon, 1998). Gross et al. (1995*b*), in ibex (*Capra ibex nubiana*), and Ginnett and Demment (1997), in giraffe (*Giraffa camelopardalis*), found that females cropped bites more quickly and chewed faster than males. Although sward height affects bite rate (i.e. lower bite rates in the taller sward in both sexes), Pérez-Barbería and Gordon (unpublished data) found that it did not mask the fact that female Soay sheep (*Ovis aries*) chews faster than male on swards of different heights.

If daily time spent chewing is similar in both sexes, the faster chewing in females would enable them to use poorer pastures than males because of (*i*) females would have higher comminution effectiveness which enables them to increase the digestion efficiency, and/or (*ii*) reduce the passage time through the gut, due to the positive relationship between passage time and particle size of food, which would enable to use a similar strategy to hindgut fermenter species (i.e. to use poorer pastures but have higher intakes per time unit at the expense of digestion efficiency). If the sex differences in bite rate were great enough to counterbalance the larger bite size of males equalling the intake rate between sexes, as for Soay sheep (Pérez-Barbería and Gordon, unpublished data), males should spend a longer time foraging per day than do females, if they are to satisfy their energetic requirements. The literature presents contradictory information to support this idea and, therefore, conclusions are difficult to draw. Males spend less time in daily foraging than females in several artiodactyls species (in waterbuck, *Kobus ellipsiprymnus*, Spinage, 1968; in gerenuk, *Litocranius walleri*, Leuthold and Leuthold, 1978; in giraffe, *Giraffa camelopardalis*, Leuthold and Leuthold, 1978; Pellew, 1984; Ginnett and Demment, 1997; in chamois, *Rupicapra pyrenaica parva*, Pérez-Barbería et al., 1997), whereas in other studies males spend more time grazing than do females (in red deer, Clutton-Brock et al., 1982), or there are no differences between sexes (in black-tailed deer, Weckerly, 1993). In a comparative study Clutton-Brock and Harvey (1983) showed that time spent foraging scaled positively with body mass for 14 species of ungulates. However, these results may reflect the confounding effect of differences in food quality between species; the largest species eat poorer diets and, therefore, investing longer periods eating.

The advantages of narrow muzzles and high bite rates

Higher bite rates enable an increase in intake rate, simply because this enables more food to be cropped per unit of time. This advantage is independent of body size. Feeding on small items of herbage within the sward is possible if the muzzle width is small enough to enable the animal to ingest the items without incorporating additional undesirable forage. When the bite size of a highly nutritious small item of food is below the allometric bite size – body size relationship, a higher bite rate would enable an increase in intake rate, optimising the use of these small items of herbage, although searching time might increase reducing bite rate. When ingesting small items of herbage it may be possible to maintain the intake rate if bite depth increases. This would increase bite size, but this strategy is limited because bite size increases with increasing leaf length up to a maximum, beyond which bite size remains approximately constant (Gordon

et al., 1996). Other morphologic bite size dimensions, e.g. mouth aperture, seems to be independent of sward characteristics (Jiang and Hudson, 1994; Gordon et al., 1996).

In this context females would combine the qualities to exploit efficiently high rewarded but small and scattered items of food within a poorer sward. Prins (1989) found that bulls (*Syncerus caffer*) lost condition when they associated in mixed herds and regained condition upon segregation, whether this was due to sexual interaction or differences in feeding ability is unknown.

Conclusions

In this paper we have emphasised the influence of body size dimorphism in explaining differences between the sexes in diet selection and sexual segregation in the Cervidae. Sexual body size dimorphism has been demonstrated to be a general rule in artiodactyls, however, lack of sexual morphological dimorphism in traits involved on intake and food mastication processing was detected after controlling for body size. Body size dimorphism generates differences between sexes in bite rates (higher in females) and forage selection ability (females having narrower muzzles), which would enable different vegetation communities or habitats use by the sexes. Nevertheless, from the results reported in the literature is not possible to define a consistent pattern of sex differences in habitat use or diets in cervids, primarily because field studies do not allow us to determine which factors cause these differences. We would, therefore, encourage more experimental tests to isolate the effects which contribute to diet selection and sexual segregation in cervids. These experiments should be conducted on grazing and browsing cervids to evaluate if species of both feeding types respond in a similar fashion.

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GROWTH, VOLUNTARY FOOD INTAKE AND DIGESTION IN FARMED TEMPERATE AND TROPICAL DEER

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Growth and voluntary feed intake (VFI) in grazing temperate farmed deer species are influenced by the feeding value of the forage and the stage of the deer's seasonal cycle. Liveweight gain (LWG) of growing red deer was greater when perennial ryegrass (0.80)/white clover (0.20) pasture was grazed at 10 cm than 5 cm surface height, but venison production by one year of age was still low. Chicory and red clover were of superior feeding value for deer than perennial ryegrass-based pastures, increasing LWG of young red and hybrid (0.25 elk; 0.75 red deer) deer during summer and autumn, due to higher VFI and to higher organic matter digestibility. Relative to perennial ryegrass, chicory disintegrated faster in the rumen, with very low rumination time and faster rumen outflow rates of liquid and particulate matter. Inputs of red clover and chicory substantially increased venison carcass weights at one year of age from grazing red and hybrid stags. In indoor pen feeding studies, red deer were shown to have a seasonal cycle of digestive function, with greater rumen mean retention time (MRT) and greater rates of rumen ammonia production in summer than in winter. It was deduced that the purpose of the digestive cycle was to maintain apparent digestibility constant as VFI increased during summer. Tropical sambar deer were also shown to have seasonal cycles in growth and VFI that were of reduced amplitude compared with red deer. Peak VFI and growth occurred in autumn and minimum VFI and growth occurred in spring. Feed conversion (kg DM eaten/kg LWG) was more efficient for sambar than for red deer, due to lower VFI and lower heat production, but there was no difference between species in digestive efficiency.

Key words: Red deer, sambar deer, forages, growth, voluntary feed intake, rumen digestion

Management of captive deer world-wide varies from year round grazing systems as in New Zealand (NZ) and Australia to intensive year round indoor husbandry systems used in China and Korea, with intermediate systems as used in the UK and Europe, employing grazing during summer and indoor housing during winter. Arctic, temperate and tropical deer, ranging from highly seasonal to aseasonal, are all now farmed. A feature common to all deer farming systems is an understanding and fulfilment of nutritional requirements.

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Growth and voluntary feed intake (VFI) in grazing farmed deer are principally influenced by two processes, the feeding value of the forage diet being consumed and the seasonal biology of the young growing deer. Seasonal growth in temperate deer was first shown in mule deer (*Odocoileus hemionus*) in North America (Wood et al., 1962; Bandy et al., 1970), with maximum liveweight gain (LWG) and VFI in summer and minimum LWG and VFI in winter. Similar seasonal patterns of growth and appetite were shown for red deer (*Cervus elaphus*) in Scotland (Kay, 1979; Suttie et al., 1983) and in NZ (Suttie et al., 1989). Seasonal biology of deer is controlled by endocrinological processes, generally in response to changing daylength and is mediated by the hormone melatonin secreted by the pineal gland (Barry et al., 1989).

Ulyatt (1973) defined feeding value as the animal production response to grazing a sole diet of fresh forage under conditions of unrestricted intake. With this definition the daily LWG of young deer fed a sole diet of fresh forage would be an index of feeding value. Feeding value is mainly controlled by the chemical composition of the forage; it is higher when plants are maintained in the vegetative than in the reproductive state, when the fibre content is lowest. All plant comparisons reported in this paper were made in the vegetative state. The absolute levels of VFI and LWG that are recorded in farmed temperate deer at any point in time thus depend on the feeding value of the diet and on the stage of the deer's seasonal cycle. This paper reports recent advances made in improving the feeding value of forages for farmed deer production and discusses these as functions of increased VFI and digestive efficiency. The influence of seasonality on the growth and VFI of tropical deer has only recently been researched and will also be reviewed.

Temperate deer production from forage diets

In many countries deer farms have been established on areas that were originally grazed by farmed cattle or sheep. In NZ predominant pastures grazed by deer are an 80:20 mix of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). These pastures grow in all seasons throughout NZ, with winter growth being very low in the south of the country and summer/early autumn growth being restricted due to hot weather. Deer have been grazing these forages since the inception of deer farming in NZ in 1969. An objective of deer farming in NZ is to grow young red and hybrid (0.25 elk: 0.75 red) deer stags to reach a minimum of 92 kg liveweight (50 kg carcass) and a maximum of 120 kg (65 kg carcass) by one year of age, to develop a venison production system from grazed forages that is both biologically and financially efficient (Barry and Wilson, 1994).

An intensive 2-year observational study of commercial red deer farms in NZ has recorded growth rates and achievement of production targets (Audigé,

1995). In consecutive years 8.7 and 14.1% of red deer stags achieved the 92 kg liveweight target by 12 months of age on conventionally managed PRG/WC pastures. Between farms, achievement of this target ranged from 0 to 41% and 0–48% in respective years. These data can be used as a baseline against which the potential of new technologies for grazing, management methods and forage species can be evaluated.

Feeding value of forages for temperate deer

Ulyatt (1973) further defined the components of feeding value as

- Voluntary feed intake (generally measured as DM eaten per day).
- Nutritive value/DM eaten.
  Nutritive value can be further subdivided into two components.
- The digestive process (including apparent digestibility, sites of digestion and mean retention time).
- The efficiency of utilisation of absorbed nutrients for the growth process (generally measured using respiration calorimetry).

Increases in the feeding value of forages for deer will be discussed as functions of these processes.

Early studies (Ataja et al., 1992) showed that growth of young stags on conventional NZ pasture was greater when the surface height was maintained at 10 cm than at 5 cm, but even at 10 cm only approx. 50% of young stags attained the target slaughter criteria by 12 months of age. This stimulated studies at Massey University on the development of alternative special purpose forages and their grazing management for deer production.

The legume red clover (*Trifolium pratense*) and the herb chicory (*Chichorium intybus*) were both highly preferred in grazing selection studies with red deer (Hunt and Hay, 1990), and were selected for study because both are deep tap rooting and can withstand summer moisture stress and because in sheep studies both were of higher *in vitro* digestibility than perennial ryegrass-based pastures. Pure swards of the two special purpose forages (plus one study with *Lotus corniculatus*) were compared against a control of perennial ryegrass-based pasture, both in grazing trials to determine relative feeding value and VFI and in indoor metabolism studies to determine aspects of the digestive process. In the grazing work, studies of calf growth were made during lactation in summer (January and February in NZ), with weaning at 3 months of age at the end of February, and also during post-weaning growth to slaughter at one year of age (March to November of the same year; autumn, winter and spring). Details of the management of these

crops are given in a related paper (Barry et al., 1998) and an account of the nutritional principles affecting deer growth and VFI is given here.

During summer, inputs of red clover, chicory and also *Lotus corniculatus* (birdsfoot trefoil) increased calf growth by approximately 20%, relative to deer grazing conventional perennial ryegrass/white clover pastures, with little difference between individual forages (Table 1). Inputs of chicory increased growth by 47% during autumn and by 10% during spring (Table 2). Corresponding increases for red clover were 26% during autumn and 14% during spring. These increases in LWG from grazing young red and hybrid deer on red clover and chicory resulted in the proportion of stags attaining the minimum slaughter criteria by 12 months of age being consistently increased to 90–100%, and carcass weight being increased by 11% (red clover) and 17% (chicory) (Barry et al., 1998). These results demonstrate that red clover and chicory are of higher feeding value for growing deer than conventional NZ perennial ryegrass/white clover pasture, with the principal advantages occurring during summer and autumn.

Indoor metabolism studies showed that relative to perennial ryegrass, chicory was of higher organic matter digestibility (OMD), that it disintegrated more rapidly in the rumen with a low rumination time and that rumen fractional outflow rate (FOR) of both liquid and particulate (i.e. solid) matter were higher (Table 3). Consequently, mean retention time (MRT) of liquid and particulate matter in the rumen was shorter than for perennial ryegrass and clearance from the rumen was faster. Similar results have been found for the digestion of red clover v. perennial ryegrass by red deer (Freudenberger et al., 1994).

Table 1

Growth of deer calves during lactation (g/day) in summer (January and February). Values in brackets are relative to grazing perennial ryegrass/white clover pasture as 100, and are an index of relative feeding value

Author	Perennial ryegrass/ white clover pasture	Red clover	Chicory	*Lotus corniculatus*
		Summer		
Niezen et al. (1993)[1]	333	433 (130)		
	331	410 (124)	385 (116)	
Kusmartono et al. (1996*a*)[2]	351		404 (116)	
Adu et al. (1998)[2]	399			485 (122)
Mean	(100)	(127)	(116)	(122)

[1]Calves all red deer; [2]Half calves were red deer and half were 0.25 elk: 0.75 red deer hybrid

Table 2

Growth of weaner red (R) and hybrid (H) stags to one year of age chicory, compared with perennial ryegrass/white clover pasture. Values in brackets are relative to grazing perennial ryegrass/white clover pasture as 100, and are an index of relative feeding value

Author	Perennial ryegrass/white clover pasture		Chicory	
	R	H	R	H
		Autumn		
Kusmartono et al. (1996*a*)	178	203	246 (138)	318 (157)
Min et al. (1997)	152	199	235 (155)	271 (136)
Mean	(100)		(147)	
		Spring		
Kusmartono et al. (1996*a*)	260	271	255 (98)	310 (114)
Min et al. (1997)	285	298	335 (118)	331 (111)
Mean	(100)		(110)	

Reasons for the higher feeding value of chicory than perennial ryegrass-based pastures can be found in their differences in chemical composition (Table 4). The faster rumen disintegration of chicory can be explained by its higher ratio of readily fermentable carbohydrate (water soluble sugar + pectin): structural carbohydrate (cellulose + hemicellulose) than perennial ryegrass. Chicory has a high content of pectin (c 10% DM; an intracellular cement), which is degraded very rapidly in the rumen. The more rapid clearance of DM from the rumen results in VFI being greater for deer fed chicory than perennial ryegrass-based pastures (Kusmartono et al., 1996*a*). The lower protein (& N) concentration in chicory does not result in lower protein absorption, as duodenal N flow/kg DOMI is similar for animals fed chicory and perennial ryegrass (Barry, 1998), due to reduced rumen ammonia absorption in the animals fed chicory. The faster rumen liquid FOR in deer fed chicory is probably a response to increased rumen osmotic pressure caused by the high ash content of this plant (Table 3).

Table 3

Kinetics of feed breakdown and outflow from the rumen in red deer fed chicory and perennial ryegrass under indoor conditions

	Perennial ryegrass	Chicory	SE
Composition:			
Dry matter (g/kg)	247	161	
Total N (g/kg DM)	30.4	26.9	
Ash (g/kg DM)	102	180	
Apparent digestibility:			
Organic matter	0.744	0.820	0.0311
NDF	0.755	0.679	0.0231
Rumen pH	6.44	5.63	
Particle breakdown efficiency:			
Eating	0.37	0.27	0.038
Ruminating	0.47	0.65	0.038
Chewing time (min):			
Eating	221	209	49.2
Ruminating	257	30	54.6
Rumen fractional outflow rate (%/h):			
Liquid	13.6	18.9	2.18
Particulate (lignin)	2.78	4.08	0.551
Particulate (ADF)	2.02	4.30	0.506
Rumen mean retention time (h):			
Liquid	8.9	6.4	0.01
Particulate (lignin)	49.0	37.7	9.61
Particulate (ADF)	52.5	27.9	4.66

From Dryden et al. (1995); Kusmartono et al. (1996*a*, 1996*b*, 1997)

When maintained in the vegetative state, the OMD of chicory is consistently high at ca. 85% throughout the year (Table 4). Perennial ryegrass/white clover pastures attain this value in spring, but decline to lower values in summer and autumn, particularly during hot weather (Table 4). Changes in metabolisable energy (ME) concentration show similar trends with time. These differences in plant OMD and ME concentration with time explain seasonal differences in feeding value, with the extra LWG from feeding chicory compared to perennial ryegrass/white clover pasture being much greater in summer and autumn than in spring (Tables 1 and 2). They also explain seasonal differences in VFI, which were 56, 26 and 15% higher for deer grazing chicory than perennial ryegrass/white clover pastures during summer, autumn and spring respectively (Kusmartono et al., 1996*a*).

Table 4

Seasonal changes in the nutritive value of perennial ryegrass/white clover pasture and chicory selected by grazing deer

	Perennial ryegrass/ white clover pasture	Chicory
Ratio of readily fermentable carbohydrate/ structural carbohydrate:		
Summer	0.26	1.39
Autumn	0.21	1.39
Spring	0.27	0.95
Organic matter digestibility (% OM):		
Summer	76.5	86.4
Autumn	77.6	85.8
Winter	85.2	
Spring	84.8	86.5
Metabolisable energy (MJ/kg DM):		
Summer	11.1	12.0
Autumn	11.1	11.8
Winter	11.4	
Spring	12.1	12.1
Protein (% DM):		
Summer	21.5	20.1
Autumn	26.8	19.9
Winter	25.3	
Spring	22.6	19.4

From Kusmartono et al., 1996*a*

Seasonal changes in growth and VFI

In order to measure seasonal changes in growth and VFI it is necessary to use a diet that does not change in composition with time, which excludes the use of fresh forage diets. Early studies in Scotland showed that apparent digestibility of a range of diets fed to deer remained constant throughout the year, despite VFI increasing substantially during summer (Milne et al., 1978). The same investigation showed that MRT in the entire digestive tract was less for red deer than for sheep, with apparent digestibility being less for deer than for sheep. This was confirmed by studies in Japan, where MRT was shorter and apparent digestibility less for *Cervus nippon* than sheep when both were fed lucerne hay

cubes (Katoh et al., 1991). Subsequent studies in NZ, involving red deer fed at the same level during winter and summer (Table 5), showed the presence of a seasonal cycle in digestive function that was independent of the seasonal VFI cycle, with increases in rumen MRT, rumen pool sizes of ammonia and volatile fatty acids (VFA) and rumen ammonia irreversible loss (i.e. production rate) in summer compared with winter. Thus, it seems that temperate deer maintain apparent digestibility constant as VFI increases in summer through increasing rumen MRT, allowing more time for microbial attack, and by stimulating rumen fermentation as shown by the increase in rumen ammonia production at constant feed intake, perhaps through increased re-cycling of salivary urea into the rumen during summer. It is possible that other temperate deer species show similar seasonal cycles in rumen digestion.

Table 5

Effect of season upon rumen digestion in castrate red deer stags fed chaffed lucerne hay

	Winter *ad lib*	Summer *ad lib*	Summer restricted
Voluntary food intake:			
Organic matter (g/kg $W^{0.75}$/d)	53	77	56
Apparent digestibility:			
Organic matter	0.640	0.632	0.642
Fibre	0.436	0.394	0.421
Rumen mean retention time (h):			
Liquid	6.1		8.1
Particulate	31.7	37.5	36.0
Rumen ammonia:			
Concentration (mg NH_3-N/l)	193	191	218
Pool size (mg/g N intake)	20.4		23.9
Irreversible loss rate (mg N/g N intake)	522		649
Rumen VFA:			
Concentration (mmol/l)	82	97	91
Pool size (mmol/100g DMI)	29	37	39

From Freudenberger et al. (1994)

Compared with temperate deer species, much less is known about seasonal cycles in tropical deer. Seasonal changes in growth and VFI of young growing red and sambar (*Cervus unicolor*) stags individually maintained in pens indoor and fed a pelleted concentrate-based diet are shown in Fig. 1 (Semiadi et al., 1995). Red deer comprise 85% of NZ's 1.8 million farmed deer, but small numbers of tropical deer species were also introduced approximately 100 years

ago. The red deer show the normal pattern of seasonality for temperate deer, with high VFI and rapid growth during summer (S), low VFI and low growth during winter (W) and a reduction in weight associated with the onset of their first rut (April and May). The sambar deer also showed a seasonal cycle, but of much reduced amplitude compared with red deer, with maximum VFI and growth in autumn and minimum VFI and growth in spring. In contrast to red deer, sambar stags showed a slowing of body growth during their first rut (May–November) but did not lose weight. There were no species differences in apparent digestibility when sambar deer and red deer were fed a range of forages in both W & S (Semiadi et al., 1994; Howse et al., 1995).

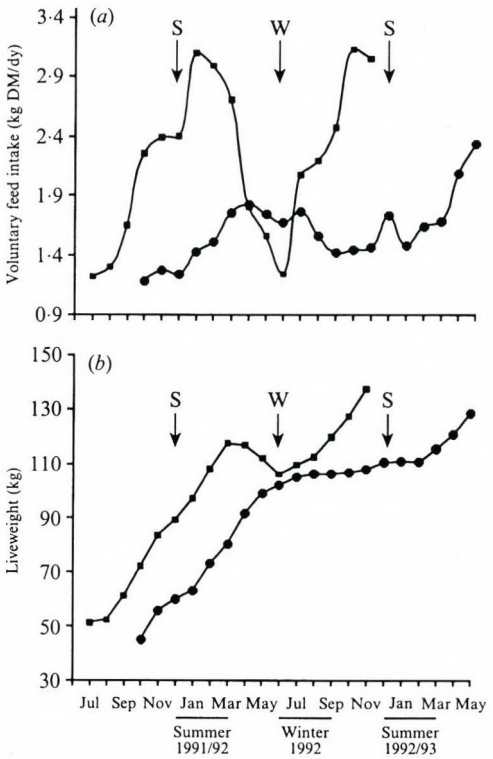

Fig. 1. Seasonal changes in (*a*) voluntary feed intake (VFI) and (*b*) liveweight in young red deer stags (temperate) (■) and sambar deer stags (tropical) (●) individually feed a pelleted concentrate diet under indoor conditions. The diet contained 29 g N/kg DM and 12.2 MJ ME/kg DM. Red deer were aged 8 months and sambar deer 6 months when the study commenced. ↓S, summer solstice; ↓W, winter solstice (Southern Hemisphere; latitude 40° S; natural daylength) (from Semiadi et al., 1995)

Growth, VFI and food conversion efficiency (FCE) of the two species fed a pelleted concentrate-based diet over a corresponding 12-month period are shown in Table 6. Averaged over the complete year, LWG was similar for the two species but VFI was substantially less for sambar deer; consequently FCE (kg DM eaten/kg LWG) was lower and hence the growth process was more efficient for sambar deer (Semiadi et al., 1995). Calorimetric studies subsequently showed that the amount of ME required for maintenance was lower for sambar deer than for red deer (0.46 v 0.57 MJ/kg $W^{0.75}$/d); the efficiency with which ME was retained above maintenance (K_g) was also higher for sambar than for red deer (0.73 v. 0.68), but in this instance the difference was not significant (Semiadi et al., 1998). The calorimetric studies show that the superior FCE of growing sambar deer over red deer is due to reduced heat production and suggests that during their evolution tropical deer adapted to hot tropical climates by reducing their level of heat production. Similar results have been found for *Bos indicus* and *Bos taurus* cattle in Australia (Frisch and Vercoe, 1969).

Table 6

Voluntary feed intake (VFI), liveweight gain (LWG) and feed conversion efficiency (FCE) (mean, SE) in young sambar and red deer, fed indoors on a pelleted diet *ad libitum*, over corresponding 12-month periods (sambar: Nov. 1991–Nov. 1992; red deer: July 1991–July 1992)

	Sambar stag	Red stag	Sambar hind	Red hind
Initial age (days)	198 (64.0)	201 (2.5)	169 (39.0)	204 (2.5)
VFI (kg DM/day)	1.63 (0.072)	2.21 (0.131)	1.42 (0.081)	1.82 (0.293)
LWG (g/day)	138 (24.5)	159 (17.8)	139 (1.3)	126 (33.2)
FCE (kg DMI/kg LWG)	12.2 (2.21)	13.9 (1.09)	10.3 (0.70)	15.0 (1.66)

From Semiadi et al. (1995)

Conclusions

Where deer production occurs from grazed forages, these studies have shown increased growth and VFI in temperate systems from the use of red clover and chicory, relative to perennial ryegrass-based pasture. The principal reason for their superior feeding value is faster breakdown in the rumen, leading to higher VFI and OMD. These or similar forages of higher feeding value should be evaluated in other temperate deer production systems. The present studies

have shown that temperate legumes are of higher feeding value for deer than grass-dominant pastures, as is well known with sheep (Ulyatt, 1973, 1981). This principle also needs to be tested with tropical deer, through studying response in VFI and LWG of deer grazing low quality tropical grasses to supplementation with tropical legumes.

The low degree of seasonality and more efficient growth of sambar deer suggests that this species should be considered for deer production systems in the tropics. Growth and efficiency of food utilisation should be studied in other deer species that evolved in the tropics.

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DEER HEALTH AND DISEASE

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This paper describes the most significant diseases of farmed deer which have emerged over the last 30 or so years. It describes their characteristic signs, how control measures have evolved, their current status and gives an indication of future diagnostic and control measures. Overall, it shows that wild deer brought into a farming environment have developed some of the production limiting diseases which affect sheep and cattle, such as parasitism and trace element deficiencies. In addition, farmed deer are susceptible to potentially fatal diseases such as tuberculosis, malignant catarrhal fever and yersiniosis. A disease which has recently emerged and has the potential to be more serious than any of the above is Johne's disease. In North America, Chronic Wasting Disease occurs in captive and wild deer in only two states but has the potential to be a serious threat to wild and farmed deer elsewhere if it spreads. The zoonotic risks of diseases affecting deer are discussed, as well as stress, welfare and deer restraint. The productivity of farmed deer can be maximised by using a well-designed deer health programme integrated with good management and feeding.

Key words: Deer, diseases, malignant catarrhal fever, tuberculosis, parasitism, yersiniosis, Johne's disease, Chronic Wasting Disease, zoonoses, restraint, stress, copper deficiency, health programmes

For scientists, veterinarians and others interested in wild and farmed deer, it has been an exciting 15 years since the first Biology of Deer Conference held in Dunedin, New Zealand.

Although there has been considerable research input over the last 50 years from wildlife scientists, especially related to control and management of deer species which are hunted and others which are endangered, there has been a dramatic increase in deer research over the last 20 years, especially in the area of productivity, health and disease of farmed or ranched deer. This is partly because of the need for more information, but also because farming has allowed better access to deer than was possible with wild or even zoo animals. At the same time, new disease problems have arisen in farmed deer which were either insignificant or uncommon in wild deer. This huge increase in knowledge of diseases in farmed deer has surely benefited wildlife scientists and veterinarians in general.

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Diseases 'yesterday, today and tomorrow'

The following diseases are those which I believe are the most important affecting farmed deer, especially in New Zealand, Australia, the United Kingdom and North America. Many of these diseases also have some relevance for wild deer. I will present them from three perspectives; looking back over the last 20 or 30 years (yesterday), here and now (today) and the likely direction of progress in the future (tomorrow). It is important to note that although the term 'deer' is used in a general sense, there are often big differences between species in terms of disease susceptibility, handling and drug efficacy. Limited space allows only some of these differences to be addressed (see also Mackintosh, 1991).

Malignant Catarrhal Fever (MCF)

This disease has been reported to occur sporadically in zoo and park deer for the last 30 to 40 years. Papers on MCF have featured in all three Deer Biology Conferences and have been the subject of plenary papers in 1990 and 1994 by Dr Reid of the Moredun Institute. It has been an enigmatic and difficult disease to investigate, due mainly to the fact that the causative agent has never been isolated or grown in culture. However, it is believed that MCF in deer in NZ, Australia and the UK is caused by a gamma-herpesvirus carried by sheep (sheep-associated or SA-MCF) recently designated Ovine herpesvirus-2 (OHV-2) which is closely related to Alcelaphine herpesvirus-1 (AHV-1), the cause of wildebeest-associated MCF in Africa and zoos around the world (Reid, 1992; Reid, 1998). Sheep are thought to be infected at or around the time of birth and can be efficient long-term carriers of the virus. Different species of deer have varying degrees of susceptibility to MCF, ranging from fallow deer which are highly resistant, through red deer which are moderately resistant and to Père David's deer which are highly susceptible. Deer appear to be 'dead-end' hosts and there is little or no deer-to-deer or deer-to-cattle transmission under natural pasture conditions. The virus is strongly cell-associated and infection can be transmitted experimentally between deer by the transfer of whole blood or tissue homogenates. The disease in deer is almost invariably fatal and there is no effective treatment. The majority of cases are very acute, with death in 1 to 3 days, and signs of erosions and ulceration of the buccal cavity, severe haemorrhagic gastroenteritis and enlarged oedematous lymph nodes. A few cases become chronic but usually die in 3 to 5 weeks, often with emaciation, crusting around mouth, nares and anus, and enlarged tortuous blood vessels (the result of chronic endothelial damage). Histopathological signs of vasculitis and perivascular cuffing are characteristic (Reid et al., 1989). The pathogenesis appears to be related to the infec-

tion of T lymphocytes, massive lympho-proliferation, deregulation of the immunomodulatory function of large granular lymphocytes and damage to endothelial cells. Epidemiologically, in deer farmed in NZ there is a preponderance of MCF cases in the winter/spring period and the annual incidence is approximately 1%, with the attack rate higher in adults than weaners and higher in stags than hinds (Fennessy, 1988). The disease appears to be precipitated by stresses such as underfeeding, exposure and social stress. Prevention revolves around minimising stress, providing shelter, feeding well and minimising contact with sheep and with pastures, feeds or clothing/objects contaminated by sheep.

The recent development of a polymerase chain reaction (PCR) test to detect OHV-2 DNA in animal tissues provides another tool for confirming the diagnosis of MCF in clinical cases and it may be useful as an epidemiological tool. Preliminary studies indicated that a number of deer appear to become latently infected for some time without showing any signs of disease (Mackintosh and Tham, 1998). The test is undergoing further evaluation for its usefulness as an epidemiological tool.

Further research is needed in the areas of epidemiology, latency, mechanisms of innate resistance/susceptibility and potential vaccines.

Tuberculosis (Tb)

Tb, caused by *Mycobacterium bovis*, has been recorded in wild and park deer in Europe and North America for over 60 years (Francis, 1958) but the disease received relatively little attention until the advent of deer farming in the 1970s and 1980s when it was recognised as a potential major threat. Since then there have been numerous papers on the subject, mostly relating to diagnostic tests, epidemiology, vaccination and control.

It appears that all species of deer are naturally very susceptible to Tb. This disease has the potential to cause great losses in infected herds if it is allowed to go unchecked, and it is also important from an international trade perspective. Up until the mid 1980s, intradermal skin testing with bovine tuberculin (purified protein derivative, PPD) was the only tool for the routine diagnosis of Tb in live deer, with the addition of histopathology and culture in dead animals. Since then the introduction of the commercial Blood Test for Tb (BTB), which is a combination of lymphocyte transformation (LT) and ELISA, has revolutionised Tb diagnosis and provided an invaluable tool for immunological an epidemiological research (Griffin et al., 1994). The PCR test, specific for *M. bovis*, is proving a useful and rapid alternative to culture. In the future, an array of new cytokine assays, including those for IL2, IL4, and gamma-interferon, and other immunological tests such as macrophage killing assays, are being developed for deer

and will provide essential tools to probe the immune system and assist in understanding the differences between protective and non-protective responses (Hook et al., 1995).

We currently have a good (although not complete) understanding of many of the epidemiological factors affecting Tb in farmed deer (Mackintosh and Griffin, 1994; Mackintosh et al., 1995*a*; Lugton et al., 1997) and some of those affecting wild deer. The predominant feature of Tb in farmed deer is the preponderance of early lesions in the tonsils and lymph nodes of the head, suggesting that the mode of transmission is via contaminated food, water or dust or by licking, nuzzling or grooming infected animals, rather than by true aerosol (lung-to-lung) transmission as occurs most commonly in dairy cattle (and humans). We have established an experimental Tb infection model which closely mimics natural Tb in terms of the lesion distribution, disease severity and immunological responses. It is based on instilling 0.2 ml of virulent *M. bovis* culture into the pharyngeal tonsil of anaesthetised deer (Mackintosh et al., 1995*b*). The immune response is monitored periodically using the BTB and the deer are usually slaughtered after 4 to 6 months to ensure that they do not suffer unduly. At this point the severity of lesions ranges from nil, through single retropharyngeal lymph node abscess, to multiple head, lung and abdominal lymph node abscesses. Using this model we have shown that there is a strongly heritable basis to natural resistance to Tb ($H^2 = 0.48 \pm 0.24$) and we are actively seeking immunological and genetic markers which would allow us to screen out highly susceptible animals and select for highly resistant herd sires in areas where infected wildlife pose a risk of Tb introduction (Mackintosh et al., 1997).

The Tb challenge model has also been used to test Tb vaccines in deer. It has been shown that two doses six weeks apart of *M. bovis* BCG vaccine gave significant protection against Tb, whether given by subcutaneous injection, by instillation into the tonsil or by aerosol into the mouth (Griffin, 1995; Griffin et al., 1995). Injection of killed vaccine with an oil adjuvant gave no protection, but stimulated a high non-protective antibody (B cell) response. Unfortunately, vaccination with *M. bovis* BCG tends to make the animal reactive to the normal Tb skin test, and therefore vaccination is unsuitable at the present time for routine use on deer farms. However, vaccination of wild deer is likely to be a useful control option if a suitable oral bait delivery system can be developed. This could be used for ring vaccinating some wild populations of red deer in NZ which have a prevalence as high as 30%.

In the future, therefore, I can foresee the development of oral vaccine baits for wild deer. Research is also being undertaken on genetically engineering BCG to improve its antigenicity and to incorporate additional antigens to act as markers to distinguish vaccinated from infected deer. New tests are being developed which detect antigens such as ESAT6, which are specific for virulent

M. bovis. Spoligotyping (van Embden et al., 1995) will be used more widely to allow more rapid typing of Tb strains for epidemiological investigations. It is hoped that immunological and genetic markers for natural resistance and susceptibility will be developed for deer which will allow selective breeding for resistance and they may also provide insight into the mechanisms which determine the differences between protective and non-protective immune responses.

Parasitism

In the wild, most deer have small parasite burdens, and serious disease or death due to parasites is unusual. This is typical of host-parasite relationships which have evolved over a long period of time in relatively stable environmental conditions. Disturbances to these conditions may result in destabilisation of these relationships. For example, an unusual reduction in feed availability or an increase in reproductive efficiency can cause relative over-population and parasite numbers can rise dramatically, resulting in disease or death. In effect, this is what happens when deer are farmed. High stocking rates and intensive grazing of fertile pastures has resulted in the same kinds of parasitic problems in farmed deer as occur in sheep and cattle. Some of the first research carried out in zoo deer and in the early days of deer farming was to learn how to overcome parasitism, especially lungworm (*Dictyocaulus* sp.) in young deer in their first autumn. Fortunately red and fallow deer develop resistance to lungworm with time and exposure, and in many farming situations it is unnecessary to treat adult red and fallow deer with anthelmintics. However, farmers soon learned that wapiti (elk), although closely related to red deer, are far more susceptible to parasites, especially gastrointestinal nematodes. During the 1980s numerous imported wapiti developed a 'fading syndrome' in New Zealand. This was characterised by chronic weight loss, with or without obvious diarrhoea, and the only consistent biochemical changes were low serum albumin and total protein concentrations. The high value of these animals and their lack of response to diverse therapy (Mackintosh and Orr, 1990; Conway, 1990) caused great frustration. (Note: Chronic Wasting Disease was ruled out by extensive histopathological examinations.) Eventually, it was shown that the early larval stages of *Ostertagia*-type parasites were causing considerable damage to the abomasal lining resulting in a rise in pH and poor protein digestion and absorption (Waldrup and Mackintosh, 1993). The pepsinogen concentrations usually stayed within the normal range and there was no specific test for the disease. Normal doses of benzimidazoles or ivermectin proved ineffective at removing these larval parasites. Subsequently it was shown that two or three times the normal dose of ivermectin or a normal dose of moxidectin, a newer potent endectocide, was

necessary to remove these larval stages and promote recovery (Mackintosh et al., 1993). It is believed that stress also plays a part in predisposing wapiti to parasitism, so that wapiti bulls after the rut in late autumn/winter and wapiti cows in late spring/summer when heavily pregnant or lactating appear to be most susceptible. It is thought that North American wapiti have not been exposed to some of the species of *Ostertagia*-type worms which infect red deer in Europe and have not evolved suitable protective responses. Wapiti in their natural environment experience very harsh environmental conditions in winter and also naturally graze longer swards which tend to have a lower parasite challenge. These factors may also have resulted in naturally lower burdens and poorer defence against the high parasite challenge experienced on farms.

Currently parasites on NZ farms are controlled by parasite monitoring, good pasture management and integrated drench programmes (see Table 1). There are numerous papers available on the efficacy of anthelmintics in deer (see Deer Branch, NZ Vet. Assoc., Courses 1, 3, 8, 10 and 11).

In the future, new tests may become available to diagnose abomasal parasites, based on the detection of faecal antigens rather than faecal egg counts, because of the difficulty detecting the pathogenic larval stages, before the infection becomes patent and eggs are shed in the faeces. There is also a general trend to find alternatives to anthelmintics in the pastoral farming industry, because of the threat of emergence of anthelmintic-resistant strains of parasites. Future research should lead to the integrated use of management, selective breeding of naturally resistant deer, the development of vaccines and the judicious use of anthelmintics for optimal control.

In North and South America, Europe and Asia there are many other parasites affecting deer including trematodes such as *Fasciola hepatica* and *Fascioloides magna*, cestodes such as *Taenia* sp. and *Echinococcus* sp., nematodes such as *Parelaphostrongylus* sp., *Elaphostrongylus* sp., *Onchocerca* sp. and *Eleophora* sp., dipterid parasites such as nasal bots (*Cephenemia* sp.) and warbles (*Hypoderma* sp.), and protozoal diseases such as cryptosporidiosis, toxoplasmosis, sarcosporidiosis, babesiosis, etc. Wild deer have largely evolved stable relationships with these parasites resulting in few mortalities. However, the introduction of new species of deer onto farms and the farming of existing species in many of these areas around the world have resulted in clinical parasitism, and various methods have been developed to cope with these problems.

Yersiniosis

Yersiniosis, caused by *Yersinia pseudotuberculosis* (and occasionally *Y. enterocolitica*), in deer is typically an enteritis and lymphadenitis and is charac-

Table 1

Typical Deer Health Programme for red deer farmed in New Zealand

1	**Vaccination:**	**Against**	**Dose**	**Age**
	Yersiniavax	*Y. pseudotuberculosis*	2 doses 3 to 6 weeks apart	weaners
	Clostridial 5-in-1	Tetanus Pulpy kidney Blackleg Malignant oedema Black disease	sensitising dose annual booster	weaners pregnant hinds
	Leptospiral triple vaccine	*L. hardjo* *L. pomona* *L. copenhageni*	sensitising and booster annual booster	
2	**Anthelmintics**			
	Benzimidazoles (B) Ivermectin (I) Moxidectin (M)	lungworm and abomasal worms	B – 3 week intervals I – 5 week intervals M – 5–6 week intervals	weaners in autumn and spring and when indicated in yearlings and adults
	Triclabendazole	liver fluke	when indicated	
3	**Trace elements**			
	Selenium	white muscle disease poor growth rate	add to anthelmintic or as pour-on, annual injection or intraruminal 'bullet'	all ages
	Copper (copper oxide wire particles, injections or topdress pasture)	osteoarthropathy, enzootic ataxia poor growth rate	winter/spring	all ages

4 **Tb testing:** Test at 6-month intervals until 3 clear whole herd tests over 2 years. Annual or biannual tests to maintain 'accredited-free' status. Skin testing with bovine PPD routine screen. Comparative cervical tests used where non-specific reactivity high. BTB to retest positive reactors. ELISA test for skin test negatives to detect anergic Tb deer.

5 **MCF:** Avoid sheep contact, feed well, minimise stress.

6 **Johne's disease:** Avoid sheep and cattle contamination of pastures, maintain closed herd or buy from low-risk sources.

7 **Miscellaneous:**

Ryegrass staggers – Avoid high endophyte ryegrass pastures in summer.

Facial eczema – Avoid grazing pastures with high sporedesmin spore counts.

Ticks – Control with appropriate management and sprays.

terised by a green smelly diarrhoea which rapidly turns bloody and results in death in one to three days if untreated. It is common in young farmed deer in their first autumn/winter or in newly captured deer, and is predisposed to by-stressors such as weaning, transport, bad weather and underfeeding. The disease has emerged as a significant cause of mortality since deer were first captured and farmed in NZ, and it also occurs in Australia, North America and Europe. Treatment with antibiotics such as tetracyclines and intensive oral or parenteral fluids is usually effective if given early enough in the course of the disease.

Today, prevention relies on good management, optimising feeding, providing shelter and minimising stress. The development in New Zealand of a killed vaccine containing strains I, II and III has proved a valuable adjunct to good management. This bacterin does not provide 100% protection but it is widely used and has reduced the number of outbreaks of yersiniosis. In the future, the development of a more sophisticated vaccine will depend on the growth of the deer industry to a size which justifies the cost of its development, which is likely to be high.

Copper deficiency

Cases of enzootic ataxia and joint problems have occurred in some wild and park deer in the past, but the exact causes have not always been identified. Farming deer has given us the opportunity to study their trace element status over a period of time, with and without copper supplementation and helped us understand their copper requirements. It has been shown that the concentrations of copper in pasture oscillate annually from a low in winter/spring to a high in summer/autumn, partly as a result of higher soil temperatures increasing soil copper availability. Silage, which is usually made from rapidly grown spring grass, tends to be very low in copper. Legumes grown in summer tend to be much higher. The absorption of copper by the animal can be affected by the concentrations of molybdenum, iron and sulphur which have the ability to form insoluble complexes in the rumen and make the pasture copper unavailable to the animal. Other factors affecting copper status include species differences in copper requirements, interactions with parasites and an apparent reduction in copper uptake with advancing age. This makes understanding copper requirements very complex.

Deer are normally born with relatively high concentrations of copper in the liver (which acts as the body's store), due to transplacental transfer. If hinds are deficient during pregnancy, the calves are born with low copper reserves, and there is little copper in milk. Under these conditions and with the rapid growth of young deer, copper deficiency can lead to osteoarthropathy including swollen 'creaky' joints and spontaneous fractures in young deer, predominantly between two and six months of age. If young deer which are marginally low in

copper survive through the autumn they are likely to become deficient in the late winter/spring period, and a proportion may develop enzootic ataxia which is a consequence of a progressive demyelination of the nerve tracts in the spinal cord. This disease is characterised by weakness of the hind leg muscles, progressing to ataxia and complete loss of use of the hind limbs in severe cases. Subclinical copper deficiency is thought to cause reduced growth rates, poor antler growth and may reduce disease resistance.

The diagnosis of copper deficiency is not simple. The liver is the main storage organ for copper and the best diagnostic sample is a piece of liver which can be taken at slaughter or by liver biopsy from live deer. Blood samples are more convenient to take but are less useful on an individual basis. They can be of value if taken at the correct time of year and if the results are interpreted correctly. Animals have homeostatic mechanisms to maintain serum copper concentrations at a relatively constant level under normal circumstances. However, when the animal becomes copper deficient and the liver reserves are very low, the serum concentration drops below normal in a proportion of animals. In farmed red deer liver concentrations below 80 μmol/kg (wet weight) are considered 'deficient' and serum concentrations below 8 μmol/ml in any individuals are an indication that the herd is deficient. Note, however, that individual variation, recent consumption of feed with high copper concentrations or concurrent infection can raise serum copper concentrations to the 'normal range' in deficient animals. The best time to sample deer is in spring when they are likely to be at their most deficient. Supplementation with copper in feed, by injections or by oral dosing with copper oxide wire particles in winter/spring is effective in preventing deficiency. Certain types of browse have higher copper concentrations than pasture and may help prevent copper deficiency.

Johne's disease

This disease, caused by *Mycobacterium paratuberculosis*, is rarely diagnosed in wild deer, but is emerging as a significant problem in farmed deer and has been reported in the UK, North America and NZ. It is an important disease because of its potential to cause death and reduced productivity, the lesions can be confused with Tb and there is growing international concern about the possible zoonotic potential of this disease.

Deer appear to be susceptible to *M. paratuberculosis* strains carried by cattle and sheep, but unlike these species, in which clinical disease commonly takes two to three years to develop, deer commonly show clinical disease at 9 to 15 months of age. Clinically there is a protein-losing enteropathy characterised by weight loss, rough coats, diarrhoea and green faecal staining around the anus

and hind legs. Subclinically affected animals are often detected at slaughter, where abscesses in abdominal and occasionally in head lymph nodes can look grossly and histologically like tuberculosis lesions, and must be differentiated by culture or PCR. Similar lesions can also be caused by *M. avium*. Serological tests such as agar gel diffusion (GD) or ELISA are useful for confirming clinically affected animals, but have only moderate sensitivity for detecting subclinically affected animals (Mackintosh and de Lisle, in press). Because eliminating carrier animals is very difficult and expensive, it is paramount that infection is not introduced into clear herds. This relies on keeping a closed herd, not intergrazing with sheep and cattle, and buying only from sources with a well-documented testing history to prove they are low risk.

In the future, it is imperative that better diagnostic tests are developed, in order to detect carrier animals. There is a need to develop better vaccines which can prevent infection without sensitising the animals to normal Tb tests.

Chronic Wasting Disease (CWD)

Although originally first discovered 30 years ago, CWD has risen to prominence lately because of the international concern with spongiform encephalopathies, especially BSE, and the possible zoonotic risks. CWD has been reported to occur in mule deer, white-tailed deer and elk, both in captivity and in the wild in Wyoming and Colorado. It has also been found in a mule deer and an elk in Canada, but tracebacks showed that both deer originated in Wyoming. CWD has the potential to become a very serious threat to wild and farmed deer and must not be allowed to spread.

CWD is characterised by ill-thrift, weight loss, teeth grinding, excess salivation and general depression. Animals tend to be hyperexcitable and some develop hind limb ataxia. There is a peak incidence at three to four years of age.

The transmissibility of CWD to other species is currently being assessed and it is not known if it poses a zoonotic risk. However, common sense would suggest that red meat from clinically normal deer is unlikely to be a danger, but offal, brain and spinal cord should be avoided as should all meat from clinically affected deer. Legislation in the UK now bans the inclusion of any meat meal of ruminant origin in feeds intended for livestock and it would be prudent for North American legislators to follow suit.

Stress/Animal Welfare

The widescale 'domestication' of deer in the last 30 years has necessitated the capture of many thousands of animals, their transport, relocation behind

fences, yarding, handling and their adaptation to a pasture diet. The stresses of this have been considerable and have resulted in high selection pressure for animals which can adapt to these changes. Cases of post-capture myopathy and stress-induced haemorrhagic enteropathy occurred in the immediate period after capture and affected animals rarely survived. Those individuals which were stress-prone, very 'flighty' or aggressive did not survive. In the early days of farming there was a high incidence of diseases with a stress component, such as MCF, yersiniosis and ulceration of abomasum and duodenum. Occasionally instances of serious outbreaks of Tb could often be associated with poor management, low productivity and stressful conditions. Since then, deer have become better adapted to pastoral farming conditions, the level of management has improved dramatically and consequently the incidence of stress-prone diseases has declined. Nevertheless, deer are still prone to stressors such as weaning, underfeeding at critical times, lack of shelter from bad weather, transport, trace element deficiencies, overcrowding and social stresses. Considerable attention is being paid by the deer industry in NZ and overseas to introduce 'quality assurance' schemes which provide assurance of high standards of farming, velvet antler removal, deer transport, deer slaughter and meat handling at all stages of 'pasture-to-plate' to ensure that the best quality product gets to the customer. Minimising stress is a crucial component of this programme, especially while yarding, during transport and in lairage. The deer transport industry has codes of conduct to ensure high standards and similarly deer slaughter plants have their own quality assurance standards which must be met before they can be certified as producers of 'Cervena' brand venison.

We have developed a stress-model using corticosteroid implants to mimic chronic stress in order to study the effect of stress on the immune system (Thomson et al., 1995). This model together with behavioural studies can be used to determine the degree of stress associated with various procedures on farms and can be used to evaluate means of reducing stress.

Zoonoses

Currently there is increased global awareness of zoonotic risks and it is sensible to consider such risks associated with farmed and wild deer. On farms, deer can be vectors of Tb, yersiniosis due to *Y. pseudotuberculosis* and *Y. enterocolitica*, leptospirosis, brucellosis, *E. coli* 0157 and parapox virus. In North America there is increasing concern over the role of deer in the transmission of Lyme disease. There are suspicions they may also carry toxoplasmosis, cryptosporidiosis, listeriosis, anthrax, rabies, tularaemia, Q fever and Rocky Mountain spotted fever in some countries.

Deer restraint

Mechanical restraint devices have been developed and improved greatly over the last 30 years, and have facilitated deer handling for routine procedures such as Tb testing, blood sampling, pregnancy scanning and *per vaginam* artificial insemination. There are many commercial devices available so I will not elaborate. Chemical restraint is now also quite refined, with a range of drugs to suit different deer species, situations and personal preference. In NZ, where the species farmed is predominantly *Cervus elaphus* species such as red and wapiti deer, xylazine is most commonly used (yohimbine antidote), often with the addition of fentanyl citrate plus azaperone or carfentanyl (naloxone antidote). For fallow deer the drug combination of choice is xylazine plus ketamine. In North America etorphine, carfentanyl, xylazine, ketamine and tiletamine are used in a wide range of applications in the wild and on farms. In the UK etorphine plus acetyl promazine, xylazine and ketamine are most commonly used for wild and farmed deer.

Integrated Deer Health Programme

The desirability of 'interfering with nature' and trying to alter any particular disease status of wild deer populations is questionable. Perhaps in some circumstances where a new disease has been introduced it may be desirable to try to stop it spreading by ring vaccinating around the infected population or by trying to eliminate isolated groups of infected animals. Otherwise opportunities for 'improving the health' of wild deer are somewhat limited.

The situation with farmed or ranched deer, on the other hand, is completely different. The objective of most farmers is to maximise productivity and minimise wastage. To do this efficiently the farmer should invest in a well-designed animal health programme integrated with management and seasonal patterns of feed availability and reproduction. This should include fundamental elements such as an investigation of the trace element status of the farm using soil, pasture and animals samples, monitoring of parasite burdens, assessment of vaccine requirements, Tb testing to fulfil statutory and the individual needs of the farmer for Tb control and a combination of policies and procedures to prevent the introduction of diseases not already present. Table 1 summarises some of the key elements in a typical NZ deer health programme. However, ultimately the single most important factor in deer health is optimal nutrition.

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THE WELFARE OF DEER

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Recent research on the welfare of farmed deer, as for other species, has addressed issues relating to transport, housing and slaughter. While most studies have continued to use a behavioural and physiological approach, new methods of assessing welfare need to consider aspects of cognition and awareness, which are not so easily assessed. It is suggested that a systems-based analysis may be one way to obtain a view of welfare from an animal-centred position. Incorporating an economic perspective could indicate whether consumer choice or legislation would be more likely to lead to higher standards.

Key words: Welfare, stress, deer, behaviour, physiology

At the last Congress, Goddard and Matthews (1997) considered the issues of stress and animal welfare and presented a review of the techniques currently available to measure, in particular, behavioural and physiological indicators of stress. They suggested that technical advances would facilitate physiological studies and that there would be a move towards studies of preference and motivation and a cost-benefit analysis of public decision making. This paper will attempt to identify whether such changes in emphasis have occurred. Although much of the work since that time has been conducted in farmed species other than deer, it is useful to draw on these studies to consider the implications of the work for farmed deer.

Animal welfare research should inform debate aimed at resolving potential conflict between production or management systems and standards acceptable to the public, as expressed, for example, by the UK Farm Animal Welfare Council's 'Five Freedoms' (FAWC, 1994). From papers on welfare-related topics across the food-animal species it is clear that certain issues, such as housing, transportation and slaughter, have received the most specific attention in recent years. This also applies to research on farmed deer. A number of other issues are emerging in relation to deer welfare which go beyond the farming environment and relate to, for example, hunting or the management of wild populations of deer.

New methods of assessing animal welfare are being developed which consider the animal's own view of a particular situation and a number of research papers have focused on aspects of anxiety, fear or cognition. Of particular interest is the possibility that there may be a conflict between what conditions a human might consider appropriate to permit good welfare and the animal-centred

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view of the same situation. The public place a high value on animals living under 'natural' conditions yet the animals may experience environmental challenges under these conditions which cause anxiety and fear. We also need to establish how members of the public become informed about welfare issues, on what they base their opinion and the best mechanisms for information transfer to enable them to make informed decisions about animal welfare, not from an anthropomorphic but from an animal-centred perspective. Producing a more animal-centred or holistic view may be made easier if we adopt a systems-based approach, which considers an animal's lifetime experience. Integrating a large amount of data is facilitated by the use of computer-based decision support systems which are currently being developed and could be extended to the welfare of farmed deer.

What does the term 'welfare' mean?

There have been many attempts to define welfare. Duncan and Petherick (1991) suggested that welfare should be defined in terms of the awareness of the animal of its situation and that if an animal's cognitive needs are met, its physical needs will also be protected. The importance of subjective feelings was also highlighted by Dawkins (1990) in relation to the potential for mental suffering to occur, leading to animals experiencing unpleasant mental states, possibly analogous to those experienced by humans. A more wide-ranging view has been taken, for example by Broom (1986). Here the animal's state in relation to its surroundings and its ability to cope are important (Dantzer, 1994). Wiepkema and Koolhaas (1993) specifically considered aspects of predictability or controllability of environmental events as being important components of an animal's coping response. From these and other definitions it is clear that welfare is a state and not itself a direct measure. However, an animal can be positioned on a welfare scale in relation to a number of criteria. For example, by reference to the UK Farm Animal Welfare Council's 'Five Freedoms' (FAWC, 1994) it is possible to make a judgement as to whether an animal is experiencing good or bad welfare. As Broom (1988) pointed out, 'It is also desirable to be able to recognise that welfare is good by positive evidence rather than by the absence of negative evidence'. The element of (human) subjectivity in assessing and balancing attributes of welfare introduces the notion that there is an ethical dimension to welfare assessment.

Assessment of welfare

There is an increasing emphasis on the adoption of a holistic approach to assessing welfare, on the basis that if changes in a number of parameters point simultaneously to the same conclusion then inferences about animal welfare can be

drawn with greater certainty (Blackshaw, 1986; Broom, 1988; Von Borell, 1995). However, it is clear that animal welfare cannot be assessed simply (Mason and Mendl, 1993). In many studies stress has been used as a reciprocal term to welfare: as stress increases, welfare decreases (Dantzer and Mormede, 1983; Moberg, 1985). Stressors have been described (e.g. mixing unfamiliar animals or transportation) which lead to measurable behavioural and physiological responses.

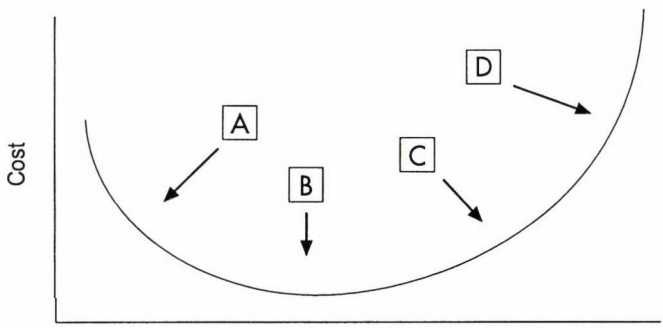
An increasing number of studies are focusing on motivation or preference, to gain an indirect assay from a more animal-centred perspective. Since welfare is related to what an animal feels, and aspects of awareness, it is important that an appropriate experimental approach is adopted which reflects this line of argument (Duncan, 1993), although we cannot measure how an animal feels directly. Motivation studies attempt to measure a strength of desire for a resource and preference/aversion studies can also be used to assess relative preference or aversion (e.g. by comparing pairs of situations — Abeyesinghe and Goddard, 1998) but provide limited information in absolute terms.

Behavioural and physiological information about animals in given situations is rapidly accumulating and this information has led to many practical improvements in welfare. In the report to the National Trust of England on the welfare of hunted deer (Bateson, 1997), a useful approach was adopted: by quantifying physiological changes which accompany situations recognised as ones in which welfare is poor or unacceptable, it may be reasonable to infer that when the same physiological changes occur in other circumstances, welfare is also poor. However, in most situations, measurable changes are less extreme and it is still a matter of conjecture at what point the welfare of animals is at risk (Barnett and Hemsworth, 1990; Mendl, 1991).

Measurement of physiological changes may be confounded by a considerable sampling artefact and approaches to overcome this have been developed (e.g. remote blood sampling — Ingram et al., 1994, 1997; Goddard et al., in press). Classically, plasma concentrations of stress hormones, notably cortisol, released following hypothalamic-pituitary-adrenocortical (HPA) activation, have been measured as an index of short-term stress. An attempt has been made to determine more long-term influences on reactivity of the HPA axis (Thurly and McNatty, 1973; Johnston and Buckland, 1976; Moberg et al., 1980) but changes have not always been consistent (Rushen, 1991). Moberg (1987) proposed a model for assessing the impact of behavioural stress on animals, which suggested that the best evidence of an animal suffering from stress was the development of a pre-pathological state, for example suppression of the immune system.

Cost benefit of animal health and welfare

There has been an increase in the use of an economic approach to resolve situations where action to increase the health or welfare status of an animal incurs a cost (McInerney, 1991). In situations where productivity or outputs are low, improving the health or welfare status of the animals may lead to an improvement in performance, the value of which outweighs the increased cost of inputs. (The corollary of this, that an increase in productivity is always accompanied by an increase in welfare, should not be taken for granted). There may come a point at which increases in animal welfare are not accompanied by a commensurate increase in productivity and, consequently, incur a cost. It is society's willingness to bear this cost which determines where, on a notional welfare scale, a production system is placed. As welfare status increases still further the cost escalates (Fig. 1). Unfortunately there are no agreed indices for the level of animal welfare and so the relationship remains conceptual. However, there is clearly a conflict for society between those members who wish to maximise welfare (whatever the cost) and those who wish to have cheaper products — with the vast majority somewhere in between. To resolve this conflict some would suggest that market forces alone can be deployed and a number of schemes have been introduced to allow consumers to pay a premium price for food from given production systems. Alternatives to direct market forces are likely to be legislative approaches.



At A, increases in animal welfare will usually result in a reduction in costs until an optimum point, B, is reached. Beyond this, increases in animal welfare are likely to result in increased costs, C. Further increases in welfare will increase costs sharply, D. (After McInerney, 1991)

Fig. 1. Cost benefit of animal welfare

Recent research relating to deer welfare

A number of areas of concern relating to the welfare of farmed deer — transport, mixing of social groups, the slaughter process, tissue removal/surgical procedures and housing and management stress — were highlighted four years ago (Goddard and Matthews, 1997). Has research progressed our understanding of these issues over the past four years? A number of studies have considered aspects of transportation (Grigor et al., 1997*a*, 1998*a*, 1998*c*; Waas et al., 1997). Behavioural and physiological changes accompanied transportation, indicating that transport was both physically and psychologically stressful for deer. Transport on winding roads was more physically demanding, with a greater risk of injury. However, most changes were short-lived and reversible, and a partial adaptation to transportation occurred as journey time increased, suggesting that, overall, deer welfare was not significantly compromised by the aspects of transport investigated, although journey times should be kept to a minimum. As an alternative approach to measurement of behavioural and physiological changes during actual or simulated transport, the relative aversiveness of specific procedures, such as transport or restraint, can be assessed. Using latency to enter and time to move down a raceway, Grigor et al. (1998*b*) showed restraint and transport to be the most aversive of a range of treatments imposed.

Public disquiet has been expressed about slaughtering and the results of two studies were published. Grigor et al. (1997*b*) suggested that, following a three hour road journey, for the animals' welfare it would be preferable to slaughter them as soon as possible after arrival at the abattoir. Jago et al. (1996) found that bruising was related to transport, season and animal factors and concluded that deer welfare (and carcase quality) could be enhanced by careful driving, by only transporting animals in good condition and avoiding transporting males during the rut. Other studies of relevance to slaughterhouse practice have considered raceway design for deer: deer prefer to enter wide (1.5 m) than narrow (0.5 m) races, and enter curved races more rapidly (Grigor et al., 1997*c*). The reluctance of deer to drink in unfamiliar surroundings, even when they have a water deficit, was demonstrated by Hargreaves and Matthews (1995) indicating the importance of careful facility design.

Housing conditions are also important. Pollard and Littlejohn (1996) studied the effect of pen size on behaviour of farmed red deer stags confined to yards. Based on the degree of aggression and stepping activity it was concluded that small pens were favoured in the spring and larger pens in the summer, although greater inter-individual distances and reduced pacing activity were observed in larger pens regardless of season. Pollard and Littlejohn (1998) also considered behavioural and physiological responses of weaned hinds following winter housing and showed indoor confinement to be associated with a greater inci-

dence of aggression, compared to outdoor confinement. Although there has been a trend away from handling deer under reduced lighting conditions, Pollard and Littlejohn (1995) suggested that stress during restraint was reduced under dim lighting conditions and that the provision of reduced lighting during, for example, transport, lairage or stunning should be investigated. The provision of visual cover to deer in paddocks has been shown to reduce inter-animal aggression (Whittington and Chamove, 1995) and leads to fewer disturbance reactions (Herrmann, 1995). Thus deer would appear to benefit from aspects of environmental enrichment. Weaning was shown to be less traumatic for calves if they had fence-line contact with their mothers (Haigh et al., 1997). Repeated mixing of groups of red deer yearlings was shown to affect immunological responses (Hanlon et al., 1995) and the stress response to handling was reported by Carragher et al. (1997) who, using a range of measures, showed deer well-being not to be unduly compromised by three common handling procedures.

Emerging issues

For many farmed species, quality assurance schemes have been developed.

Animal welfare aspects of these schemes should aim to address issues from an animal-centred and not simply an anthropomorphic point of view. For example, consideration should be given to environmental enrichment as noted above. While most scientific work has focused on farmed deer, the welfare of wild animals or semi-managed populations is increasingly being addressed, mostly by public debate. The publication of a report on the welfare of hunted deer (Bateson, 1997) and a more detailed analysis of the physiological effects of hunting deer (Bateson and Bradshaw, 1997) provoked considerable interest (e.g. Mason, 1998) and showed that even when a large amount of scientific information was assembled, opinion could still remain divided. The management of wild populations of deer (e.g. McShea and Rappole, 1997), immunocontraception and the treatment of sick individuals, later to be returned to the wild, are further issues which engender much debate.

What is the public perception of welfare of animals?

Most deer farming systems are perceived as likely to lead to a high level of animal welfare due to their 'naturalness' but what exactly do the public look for in such systems? Freedom to engage in a wide range of behaviours exhibited by wild relatives or ancestors of farmed species may not be a strictly relevant criterion in relation to an animal's adaptive capacity (Deag, 1996). It is not clear

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how the public rank different welfare attributes, although some clues are given by studying the choices made when purchasing products with different welfare 'labels'. A better understanding of public attitudes is needed. As has been pointed out (Sørensen, 1997), there is a very real danger of miscommunication of the reality of production systems to the consumer, particularly as urbanisation increases.

Approach for the future

In order to advance the assessment and understanding of welfare issues, one useful approach is to use a systems framework. This represents the overall procedures involved, including aspects of quality and sustainability, and could easily incorporate aspects of animal welfare. There have been a number of initiatives that have attempted to assess animal welfare at a farm level, mostly with respect to intensive or housed conditions, although even here there have been difficulties in making comparisons between systems (Bracke and Metz, 1997; Sandoe et al., 1997). It appears more likely that the approach could identify where welfare improvements could be made 'within' specific systems, by introducing elements of best practice or identifying where particular weaknesses occur. While it is necessary to develop a clear strategy for the assessment of the welfare of animals in extensive systems, it is not clear whether we can use the methods developed largely to study intensive production issues (Goddard and Matthews, 1997). It has often been the case that specific issues (e.g. deantlering) have been well studied but for 'lifetime' assessments no clear methodology has emerged. A more systems-based approach could lead to the development of an index system for on-farm assessment of welfare.

The important interactive role played by the stockman is becoming widely recognised, particularly if the animals are subjected to aversive procedures whenever they are handled: animals handled aversively develop subsequent aversion to handlers (Hemsworth et al., 1989; Boivin et al., 1994; DePassille et al., 1996). Too low a level of human contact, particularly at key ages, may result in animals being more fearful of humans (Matthews et al., 1997). This will affect the ease with which periodic inspections are made (there may be a greater flight distance) and the responses of animals at times of close handling (e.g. for routine veterinary treatment or management activities) may be exaggerated. There is thus a greater chance of injury to both animals and human handlers which clearly has important consequences for extensive systems under which most farmed deer are managed. It may be possible to eliminate these adverse responses to humans by developing a targeted handling regime at key 'sensitive' periods (Le Neindre et al., 1996) or to select individual animals that respond in a more appropriate manner.

The implementation of enhanced standards of welfare will depend upon an effective transmission of information in an understandable format to the public and welfare legislators. Compared to setting standards for intensive production systems, determining the standards to be adopted for deer farming (like other extensive systems) is a potentially more difficult task, especially when cognitive aspects of welfare are addressed.

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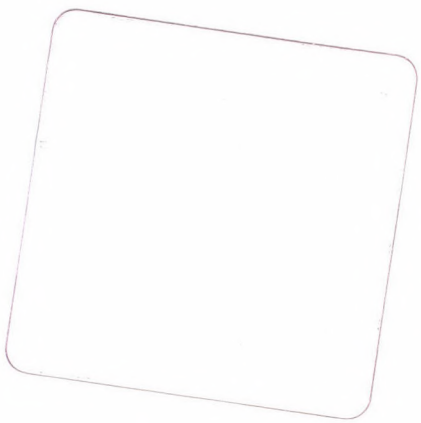

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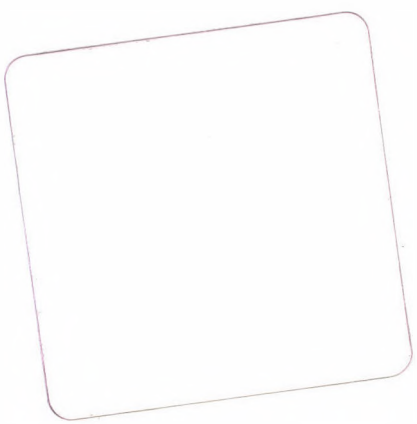

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OCCURRENCE OF SOME VIRUSES AND BACTERIA INVOLVED IN RESPIRATORY DISEASES OF RUMINANTS IN HUNGARY

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Serological surveys, virological and bacteriological investigations were carried out on large populations of lambs and calves kept in intensive fattening units on Hungarian large-scale farms, in order to study the occurrence of viruses and bacteria involved in the respiratory disease complex. The investigations were carried out in stocks where 5–20% of the young animals succumbed to acute or chronic pneumonia every year. Serological surveys revealed that seropositivity to bovine adenovirus type 2 (BAV-2) and parainfluenza type 3 (PI-3) were the most widespread viral infections both in cattle and sheep (BAV-2: 56% and 88%, PI-3: 76% and 41%, respectively). Antibodies against infectious bovine rhinotracheitis virus (BHV-1) were also found in a high proportion (45%) of sera of 3–8 month old calves, while in 2–6 month old lambs ovine adenovirus 1 (OAV-1) was the third most frequent virus (22%). Bacteriological investigations demonstrated secondary infections caused by different *Pasteurella haemolytica* and *Pasteurella multocida* serotypes in most cases. In cattle herds *P. multocida* A proved to be the predominant bacterium: it was isolated in each herd examined, while *P. haemolytica* A1 strains were isolated in 7 and *Haemophilus somnus* in 2 out of 13 herds. In sheep flocks *P. haemolytica* was the most frequent species isolated, 10 serotypes were cultured from pneumonic sheep but serotypes A2, A1 and A8 were the dominant ones.

Key words: Cattle, sheep, respiratory diseases, adenovirus, parainfluenza-3 virus, reovirus, infectious bovine rhinotracheitis, IBR, *Pasteurella multocida, Pasteurella haemolytica, Haemophilus somnus,* Hungary

In the last decades concentration of livestock was seen in the cattle and sheep husbandry of Hungary. The accumulation of animals on large-scale farms led to severe outbreaks of respiratory diseases which sometimes caused huge economic losses.

In the case of enzootic respiratory diseases virological and serological examinations proved the importance of various viruses as initiative agents of the acute phase of the disease. Secondary bacterial infections led to chronic pneu-

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monia, which was one of the main causes of mortality and cull on large-scale farms (Jensen et al., 1976; Yates, 1982; Dinter and Morein, 1990).

Up to now bovine adenovirus types 2, 4, 5, 6 and 8 (BAV-2, 4, 5, 6, 8) bovine herpesvirus type 1 (BHV-l), mammalian reovirus types 1 and 2 (Reo-1, 2), parainfluenza virus type 3 (PI-3) and respiratory syncytial virus (RSV) strains have been isolated from respiratory diseases in Hungarian cattle herds; while from sheep showing respiratory symptoms bovine adenovirus type 2 (BAV-2), ovine adenovirus types 1, 4 and 5 (OAV-1, 4, 5), mammalian reovirus types 1 and 2 (Reo-1, 2) and parainfluenza virus type 3 (PI-3) strains were isolated and the presence of the above viruses was also demonstrated by serological surveys (Áldásy et al., 1964; Bartha et al., 1974; Köves et al., 1983; Bartha et al., 1986; Tanyi et al., 1990).

Sometimes as primary but mainly as secondary agents different bacteria (*Chlamydia, Mycoplasma, Streptococcus, Corynebacterium, Klebsiella* species, *Haemophilus somnus,* etc.) are included in the pathogenesis of pneumonia of ruminants but the most severe lesions are caused by *Pasteurella haemolytica* and/or *P. multocida* (Yates, 1982).

Though these agents have been demonstrated in ruminant livestocks by direct and indirect diagnostic methods, their occurrence, prevalence and therefore significance in the pathogenesis of chronic respiratory syndrome may vary. Our aim was to investigate the occurrence of these agents in Hungarian large-scale farms where respiratory diseases were detected. Because of the common pathogens involved and the similar pathogenesis of the respiratory diseases of cattle and sheep it seemed to be worthwhile to present the results in one paper.

Materials and methods

Animals

The investigations were carried out in a period of eight years in 10 sheep- and 13 cattle-keeping farms, situated in different regions of Hungary. The first part of the examination period was dominated by sheep samples, and the second by samples originating from cattle. Most of the farms were breeding both cattle and sheep, but the animals were separated and had only indirect contact (via personnel, vehicles, etc.). The number of animals per farm ranged between 250 and 2000 (cattle) and 300 and 4000 (sheep). The age of the tested animals varied between 1 and 8 months in the case of bacterium and virus isolation, while the serological examinations included 3–8 month old calves and 2–6 month old lambs. Neither the sampled animals nor their mothers were vaccinated with any commercial vaccine against pathogens of respiratory diseases. Each farm had losses due to pneumonia for years prior to our investigations, the rate of mortality and emergency slaughter

due to that reason varied between 5 and 20%, and in most cases a sudden peak of fatalities prompted the owners or the local veterinarians to start aetiological investigations.

Virological examinations

Mediastinal lymph nodes and lung samples of 25 succumbed calves and 11 sheep were homogenized in phosphate-buffered saline (PBS). Nasal swabs collected from 378 calves and 179 sheep were treated with the same solution. Suspensions of tissue homogenates and nasal swabs were centrifuged at 10,000 *g* for 20 min to settle bacterial contaminants, and the supernatants were used for virus isolation carried out on secondary cell cultures of lamb or calf testicle and kidney, depending on the source species. Isolated viruses were identified and serotyped according to standard methods (Hoskins, 1976).

Serological examinations

Adenovirus serogroup-specific antibodies were identified by the agar gel precipitation test (AGP), using 'ana partes' mixtures of serotypes belonging to the same bovine adenovirus subgroup as antigens (Bartha, 1969).

Adenovirus serotype-specific, BHV-1, reovirus, RSV and PI-3 virus specific antibodies were detected by the virus neutralization test (VN). In the case of adenoviruses, BHV-1 virus and reoviruses the prototype strains (provided by Dr. A. Bartha, Veterinary Research Institute of the Hungarian Academy of Sciences, Budapest), in the case of RSV and PI-3 virus local isolates (Köves et al., 1983; Bartha et al., 1986) were used in the VN tests.

A total of 701 serum samples including 399 collected from cattle and 302 from sheep were examined serologically. Usually 5–10% of the herds was sampled.

Bacteriological examinations

Samples were taken from the lungs of 32 succumbed calves and 19 sheep and nasal swabs were collected from diseased animals (401 calves and 145 sheep) in 13 cattle herds and 6 sheep flocks. They were inoculated on blood and cooked blood agar plates containing 0.5% yeast extract. Blood agars were incubated at 37 °C for 24–48 h, cooked blood agars at 37 °C for 72–96 h in an atmosphere containing 10% CO_2. Isolated bacteria were identified using standard methods, *P. haemolytica* strains were serotyped by the indirect haemagglutination (IHA) test, while A and D serotypes of *P. multocida* strains were determined by the IHA test or by hyaluronidase and acriflavine tests (Biberstein, 1978; Barrow and Feltham, 1993; Carter, 1984).

Results

Virus isolation

Nine viruses were isolated during the investigation period from nasal swabs of clinically ill and from the organ homogenates of succumbed animals (Table 1). Viruses were isolated usually from more than one animal of the tested group. Dual infection (PI-3 and BAV-2) was proved by virus isolation in one industrialised sheep-keeping unit, where about 60% of 6–8 weak old lambs showed symptoms of acute, severe pneumonia. The two different viruses present in the livestock were not isolated from the same animal at the same time. In this case 20 out of 400 lambs were sampled, and six of the nasal swabs contained PI-3 virus, and another four BAV-2.

Table 1

Viruses isolated from nasal swabs and organ samples
(number of virus isolations/animals sampled in the flock or herd)

Virus	Cattle	Sheep
PI-3	1 (4/15)	1 (6/20)
BAV-2	1 (2/8)	2 (4/20)(6/12)
BAV-4	1 (3/6)	
BAV-6	1 (2/5)	
BHV-1	1 (2/10)	
Reo-2		1 (2/11)

Virus-serological surveys

The distribution of seropositivity against different viruses is shown in Tables 2 and 3.

Of the 13 investigated cattle herds, twelve were infected with PI-3 virus (75.9%), eight with BAV-2 (55.9%), seven with IBR (44.9%), three with BAV-6, two with BAV-4 and only one with Reo-2 virus. All other bovine adenovirus and mammalian reovirus serotypes were included in the tests, as well as RS, but no seropositivity was found against these viruses.

There was only one herd infected with only one of the viruses investigated. Multiple infections were far more frequent: the farms were infected with two (4 farms), three (7 farms) or four viruses (1 farm).

All sheep flocks were infected with BAV-2 causing a seropositivity rate of 88.4%. Positivity against PI-3 virus was less than half of that rate (41.1%), and that against the third most frequent virus, OAV-1 was only a quarter of it (22.2). No seropositivity was found against other sheep adenovirus and mammalian reovirus serotypes. Multiple infections were frequent in the sheep flocks

too: while only one flock was infected with one virus, five flocks with two, three with three and one with four viruses out of the investigated nine serotypes.

In both species the highest seropositivity was found against PI-3 and BAV-2: more than half of the flocks and herds were infected with these viruses, and with the exception of one herd and one flock all stocks surveyed were infected with more than one virus.

Table 2

Seropositivity against different viruses in sera collected in the different cattle herds (positive/total)

Code of herd	PI-3	BAV-2	IBR	BAV-6	BAV-4	Reo-2
Cs	11/20		20/20			
Dv	48/57	45/57	23/57			
Hn	12/15	9/15				
Jszl I	29/37					
II	36/41	28/41				
Ksz	32/48	20/48	48/48			
Lk	54/67	50/67	45/67			
Lt	10/15	7/15		4/15		4/15
Mg*	22/42	42/42		8/42		
Mf	7/10	10/10	7/10			
Nyl		12/12			8/12	
Pfsz	20/20		11/20		8/20	
Szgk	22/27		25/27	16/27		
Total	303/399	223/399	179/399	28/399	16/399	4/399
Percentage	75.9	55.9	44.9	7.0	4.0	0.9
Herd	12/13	8/13	7/13	3/13	2/13	1/13

*Farms keeping both cattle and sheep

Bacteriological examinations

Various combinations of *P. haemolytica* and *P. multocida* serotypes could be isolated from the nasal discharge of cattle showing symptoms of pneumonic disease or from the lungs of calves that succumbed to pneumonia. *P. haemolytica* serotype A1, sometimes A2 together with *P. multocida* strains were isolated from pneumonic animals in 7 farms, from 2 herds *H. somnus* and *P. multocida* A strains were cultured, while in 4 herds only *P. multocida* (mainly of type A) strains were found in the nasal discharge or in the lung samples of animals.

In sheep several *P. haemolytica* and *P. multocida* serotypes were detected. In the smaller flocks 1 or 2 *P. haemolytica* serotypes could be isolated together with 1 or 2 *P. multocida* serotypes, but in large flocks 5–10 *P. haemolytica* serotypes could be cultured together with *P. multocida.*

Table 3

Seropositivity against different viruses in sera collected in different sheep flocks (positive/total)

Flock	BAV-2	PI-3	OAV-1	Reo-2	OAV-4
B	23/30				17/30
Pfsz	22/22		15/22		
G	25/28	2/28	7/28	21/28	
Mg I*	37/43	32/43			
II	20/20	17/20	7/20		
III	22/25	16/25			
Mf	25/25				
O	28/36	7/36		11/36	
Sze	45/53	39/53	38/53		
Vt	20/20	11/20			
Total	267/302	124/302	67/302	32/302	17/302
Percentage	88.4	41.1	22.2	10.6	5.6
Flocks	10/10	7/10	5/10	2/10	1/10

*Farms keeping both cattle and sheep

In cattle herds *P. multocida* A proved to be the predominant bacterium isolated from pneumonic diseases. The majority of the isolated *P. haemolytica* strains belonged to serotype A1.

In sheep flocks *P. haemolytica* was the most frequent species isolated: 10 serotypes were cultured from pneumonic sheep, with serotypes A2, A1 and A8 being the dominant ones (Table 4). The pathologic role of mycoplasmas was shown in 9 out of 13 cattle herds and 3 out of 6 sheep flocks (data not presented).

Discussion

The aim of this survey was to investigate the incidence rate of different viruses and bacteria on large-scale farms experiencing sometimes considerable losses due to the respiratory disease complex. By the simultaneous use of the methods of virus isolation, virus-serological investigation and bacterium isolation the full scale of endemic viruses and bacteria was demonstrated on a certain farm. This method was more effective in detecting the maximum number of endemic microbes present on a certain farm than isolation alone. Presence of some other microorganisms playing a well-defined role either as immunosuppressive or as causative agents in the pathogenesis of chronic respiratory disease of ruminants (i.e. bovine viral diarrhoea virus, bovine herpesvirus type 4, mycoplasmas, chlamydia, etc.) was not studied regularly and, thus, the results of these investigations are not incorporated in the present paper.

Table 4

Isolation of *Pasteurella haemolytica*, *Pasteurella multocida* and *Haemophilus somnus* from lungs and nasal swabs of cattle and sheep (number of isolates/number of positive herds or flocks)

	No. of samples /herds	*Pasteurella haemolytica*											*P. multocida*			*H. somnus*
		A1	A2	A5	A6	A7	A8	A9	A12	A13	A16	A17	N. t.	A	D	N. t.	
Cattle lung	32/7	3/3	–	–	–	–	–	–	–	–	–	–	–	14/4	–	1/1	4/2
Cattle nasal swab	401/13	20/6	7/3	–	–	–	–	–	–	–	2/1	–	6/1	187/13	5/1	16/2	7/2
Sheep lung	19/3	5/1	1/1	1/1	1/1	1/1	3/2	2/2	–	–	–	–	1/1	4/2	3/1	–	–
Sheep nasal swab	145/6	7/2	16/5	5/2	6/3	1/1	9/3	5/2	1/1	2/2	–	3/2	2/2	20/6	1/1	3/1	–

N. t. = not typable

The results of these 'two-way' investigations (serology + isolation) of nonvaccinated weaned animals helped us to 'rank' the different microbes based on isolation and the ratio of seropositive animals. In this period calves and lambs are highly susceptible to infections, because they are already deprived of maternal antibodies.

PI-3, BAV-2, BHV-1 and BAV-6 were the most frequent viruses in the investigated calves, while lambs were infected mainly with BAV-2, PI-3 and OAV-1 viruses. Seropositivity against PI-3, BAV-2 and Reo-2 was characteristic of both species, and though there is no evidence of it, the possibility of cross infections cannot be excluded. Although certain differences were observed in the molecular structure of BAV-2 isolates, and on the basis of these findings the variants were grouped into subtypes A and B, the groups were not species specific (Belák et al., 1983). No differences have been observed between ovine and bovine PI-3 and mammalian Reo-2 isolates, which were also demonstrated in both species.

Based upon the results of experimental infections carried out with different BAV-2 subtypes (Rusvai et al., 1993) we concluded that the high ratio of seropositivity did not always correlate with the aetiologic significance of a certain virus. Therefore, despite the more frequent occurrence of BAV-2, we consider BHV-1, BAV-6 and BAV-4 to be more important in the pathogenesis of the respiratory disease complex of cattle.

In our examinations *P. multocida* A and *P. haemolytica* A1 strains proved to be the most important bacteria causing pneumonia in cattle, however sometimes other *P. haemolytica* and *P. multocida* serotypes could be isolated from the nasal discharge of diseased animals. Some authors found *P. haemolytica* more frequently than *P. multocida* (Frank, 1986; Yates, 1982). Pneumonia due to *H. somnus* occurs only sporadically, however the losses in a certain farm can be considerably high, as described also by others (Humphrey and Stephens, 1983).

In sheep herds *P. haemolytica* strains seemed to be more important as far as pneumonia was concerned. Several serotypes were involved in the pathogenesis, and our results were in good agreement with those of other authors (Gilmour et al., 1991), however the proportion of the *P. haemolytica* serotypes slightly differed from the serotyping results of *P. haemolytica* strains isolated in diagnostic institutes at postmortem examinations (Fodor et al., 1984). According to our data and other observations (Köves et al., 1982; Jensen et al., 1976) in some cases mycoplasmas can facilitate the development of pneumonia in ruminants.

Commercial vaccines containing different viruses in various composition are on market all around the world to prevent the economic losses caused by these infections. Serological investigations prior to vaccination are usually aimed at examining which viruses are involved in the respiratory disease complex on a certain farm, bacteriological examinations are performed to isolate the

causative agent(s) and to determine the antibiotic resistance of the isolated bacteria. The results facilitate the correct choice of vaccines and antibiotics. On the basis of our findings presented in this paper it can be concluded that in certain cases when the composition of the local microflora makes it possible and necessary, home-made, farm-specific vaccines containing viral and bacterial components should applied (Fodor and Rusvai, 1993). Reduction of economic losses by these vaccines suggests that combined (virus + bacterium) vaccines may prove to be a useful tool in the control of the respiratory disease complex of cattle and sheep.

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DETECTION OF *LAWSONIA INTRACELLULARIS* IN HUNGARIAN SWINE HERDS BY POLYMERASE CHAIN REACTION

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Pooled faecal samples and/or intestinal contents from pig carcasses were collected from 11 Hungarian swine farms and subjected to DNA extraction/purification and subsequent polymerase chain reaction (PCR) in order to detect *Lawsonia intracellularis*, the aetiological agent of the porcine proliferative enteropathy complex. Specific PCR positivity was detected in 6 individual and 22 pooled samples out of 46, originating from eight herds. The PCR products of collected faecal samples hybridised in Southern blot hybridisation with the DNA of the type strain *L. intracellularis* NCTC 12657. This is the first confirmed detection of *L. intracellularis* by PCR in Hungary.

Key words: *Lawsonia intracellularis*, porcine proliferative enteropathy complex, PCR, Southern blot hybridisation

Porcine proliferative enteropathy complex (PPE) is an economically important disease of swine with world-wide occurrence. Generally, the condition is characterised by adenomatous proliferation of immature epithelial cells in the ileum, sometimes in the caecum and in the proximal part of the spiral colon. The basic, uncomplicated form is called porcine intestinal adenomatosis. Three additional forms of the disease have been described, based on characteristic secondary changes such as severe bowel haemorrhage (porcine haemorrhagic enteropathy), diffuse coagulation necrosis of the intestinal epithelium (necrotic enteritis), and thickening of the muscle layer of the terminal ileum (regional ileitis) (Jubb et al., 1993). The condition usually affects pigs over 6 weeks of age, most commonly fatteners. It is associated with decreased fattening performance, chronic weight loss, runting, and sometimes sudden death. As demonstrated recently, the disease is caused by *Lawsonia intracellularis*, an obligately intracel-

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lular, microaerophilic, curved, non-spore-forming, non-motile bacterium (McOrist et al., 1995). It can be detected by a modified Ziehl-Neelsen staining in faecal smears, by histopathology using silver impregnation, by immunofluorescence or immunohistochemistry with monoclonal antibodies, and by *in situ* hybridisation or polymerase chain reaction (PCR) using specific oligonucleotides (Jones et al., 1993; McOrist et al., 1994; McCormick et al., 1995; Jensen et al., 1997). A PCR technique was used for the *ante mortem* confirmation of *L. intracellularis* infection of swine herds in several recent studies (Holyoake et al., 1996; Lanza et al., 1996). Our aim was to detect *L. intracellularis* by this PCR technique in Hungarian swine herds where the presence of the pathogen had been suspected upon characteristic clinical or pathological signs but had not been confirmed.

Materials and methods

Faecal samples. Three faecal samples of approx. 1 ml were collected from the intestinal tract of pig carcasses with suspected PPE, and were stored in plastic tubes at –20 °C until used. Pooled faecal samples from the floor of selected grower cages or finisher pens were collected in clean plastic gloves. They consisted of at least three different samples from the same pen or cage. Pooled faecal samples were immediately transported in a cooling box to the laboratory and were stored at –20 °C until used.

Extraction of DNA from faeces. Extraction of DNA from faeces was done according to the method described by Boom et al. (1990) and Jones et al. (1993). Briefly, samples (~0.2 g) were suspended in 1 ml of lysis buffer (5 M guanidine thiocyanate (GuSCN), 22 mM EDTA, 0.05 M Tris-HCl (pH 6.4), 0.65% Triton X-100), and were vortexed for 30 sec. They were allowed to stand at room temperature for 1 h and then centrifuged at 14,000 *g* for 1 min. The supernatants were transferred to a tube containing 50 µl of diatomaceous earth (DE, SIGMA-Aldrich) suspension (20% w/v DE in 0.17 M HCl). The samples were then vortexed and centrifuged at 14,000 *g* for 1 min. The pellets were washed twice in 5.5 M GuSCN and 0.05M Tris-HCl (pH 6.4), twice in cold 70% ethanol and once in acetone. The acetone pellet was dried at 56 °C for 15 min in a heating block with open lid. The DNA from each tube was dissolved in 75 µl of TE buffer. After centrifugation, the supernatant which contained the DNA was stored at –20 °C until further processing.

Polymerase chain reaction. PCR was done according to McCormick et al. (1995) with slight modification. The following primers were used: *primer A* – 5'-TATGGCTGTCAAACACTCCG-3' and *primer B* – 5'-TGAAGGTATTGG-TATTCTCC-3' (Gibco BRL, Life Technologies). Five µl sample aliquot was mixed with 10 µl PCR reagents resulting in the following final concentrations:

1 × PCR buffer, 1.5 mM $MgCl_2$, 140 μM dNTP, 1 U Taq polymerase, 0.25 μM each of sense and antisense primers. The reaction mixture was incubated at 95 °C for 5 min in a DNA thermal cycler (Omnigene, Hybaid), then the reaction was continued for 35 cycles with each cycle consisting of 94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec. The negative control samples were without DNA or with DNA prepared from the faeces of healthy animals. The positive control samples contained DNA from the type strain *L. intracellularis* NCTC 12657. The reaction products were separated by electrophoresis in a 2% agarose gel stained with ethidium bromide. The size of the amplified product was estimated by comparing it to a 1 Kb DNA Ladder (Gibco BRL, Life Technologies).

Southern blot analysis. The separated DNA was transferred to a nylon membrane by capillary blotting and fixed by UV-crosslinking (Ausubel et al., 1989). Non-radioactive probe was synthesised using a DNA fragment of *L. intracellularis* type strain NCTC 12657 as template. This template DNA fragment was generated by the PCR amplification described above and purified from agarose gel by GenElute Column (Supelco, SIGMA-Aldrich). After ethanol precipitation, 200 ng of the recovered DNA was applied to synthesise a *L. intracellularis* specific probe in a linear PCR reaction using primer B only. This reaction contained 200 μM of dATP, dCTP and dGTP each, 130 μM dTTP, and 70 μM digoxigenin-11-dUTP (Boehringer Mannheim) to make a sensitive probe with 1:2 DIG-11-dUTP:dTTP ratio. The conditions of the linear PCR were: an initial 2-min denaturation at 94 °C, and 25 cycles of 25 sec at 94 °C, 30 sec at 55 °C and 90 sec at 72 °C. The final extension was 4 min at 72 °C. After 1-h pre-hybridisation, the blots were incubated in the hybridisation mixture (6 × SSC, 5 × Denhardt's Solution, 0.5% SDS, 50 μg/ml sonicated salmon sperm DNA) containing the DIG-labelled probe at 60 °C for 3 h. After two washings at room temperature (2 × and 1 × SSC, 0.1% SDS) for 20 min, the membranes were washed under higher stringency conditions (0.5 × SSC, 0.1% SDS) at 60 °C for 20 min. The positive DNA hybridisation signals were visualised as described by the Boehringer Mannheim Manual (The DIG System User's Guide for Filter Hybridization).

PCR-RFLP. The *L. intracellularis* type strain was obtained from Dr. Steven McOrist, University of Edinburgh, United Kingdom, and was validated by PCR-Restriction Fragment Length Polymorphism (RFLP) analysis. Briefly, the DNA (amplified in the same condition we used for the samples) was gel-purified using GenElute Agarose Spin Column (Supelco, SIGMA-Aldrich). After ethanol precipitation the DNA was resuspended in TE buffer and then digested with *Pst*I and *Bsm*I restriction endonucleases. The DNA fragments were separated by electrophoresis in a 2% agarose gel, were stained with ethidium bromide, and their size was estimated using a 1 Kb DNA Ladder (Gibco BRL, Life Technologies).

Results and discussion

The presence of the PPE complex in Hungarian swine herds had been suspected for many years, but an aetiologic diagnosis had not been accomplished (Vetési and Mészáros, 1992). The specific detection of the aetiologic agent, currently named *L. intracellularis,* was previously based on the silver impregnation technique. Unfortunately, this method does not allow for *ante mortem* diagnosis, requires fresh material (intestines), and is less accurate in the absence of well-visible adenomatous intestinal epithelial proliferation. The highly specific and sensitive PCR allows us to prove the infected status of an animal even in the absence of pathognomonic lesions, or in case of advanced autolysis. This method is also suitable for determining the status of a swine herd regarding *L. intracellularis* infection, and helps estimate on-farm disease prevalence.

Swine farms included in the present investigation were selected for sampling based on herd history and the presence of suspicious clinical signs, such as uneven growth in the growing or fattening phase and/or wasting and chronic diarrhoea, as well as unexplained death of fattening pigs or replacement gilts with severe bowel haemorrhage. The examined farms are located in the eastern (5), southern (5), and western (1) regions of Hungary. A herd was termed infected when one sample was positive on PCR examination. The DNA of *L. intracellularis* was detected in 28 (6 individual, 22 pooled) faecal samples originating from 8 different locations. Positive cases showed the characteristic product of 329 base pairs in agarose gel electrophoresis (Fig. 1). The intensity of the bands varied greatly among positive cases due to the different *Lawsonia* DNA concentration in the samples. We observed less non-specific reaction by increasing the stringency of the PCR reaction reported by McCormick et al. (1995). Elevating the PCR annealing temperature from 55 °C to 60 °C and decreasing the $MgCl_2$ concentration from 2 mM to 1.5 mM in the reaction mixture greatly enhanced specificity. No difference in sensitivity was found under the higher stringency conditions (data not shown).

The identity of the type strain *L. intracellularis* NCTC 12657 was verified by PCR-RFLP analysis. Based on the reported sequence data (GenBank accession number: L08049), two restriction endonucleases were selected, which cut the 329 bp long amplified DNA at position 109 (*Pst*I) and at position 262 (*Bsm*I). After the amplified DNA was digested, the subsequent gel electrophoresis revealed a 109/220 bp or a 262/67 bp pattern of the *Pst*I or *Bsm*I enzymes, respectively. Based on the results of our PCR-RFLP analysis, the amplified DNA from the obtained source was confirmed to be *L. intracellularis* specific DNA (data not shown).

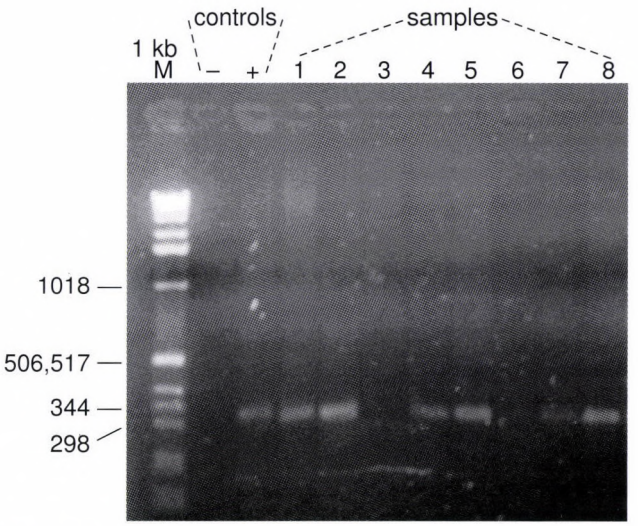

Fig. 1. PCR amplification of *Lawsonia intracellularis* DNA extracted from faecal samples of a PPE-affected pig herd. Lane 1: molecular weight marker – 1 Kb DNA Ladder, Gibco (M); lane 2: negative control; lane 3: positive control from lysed *L. intracellularis* DNA (type strain: NCTC 12657); lanes 4–11: samples of DNA extracted from faeces. Samples 1, 2, 4, 5, 7 and 8 show amplification of the 329 bp *Lawsonia*-specific DNA and were determined positive, while samples 3 and 6 show no amplified product and were considered negative

To validate our results further, the amplified DNA fragments were analysed by subsequent Southern blot hybridisation with a probe synthesised from the original DNA from *L. intracellularis* NCTC 12657 (Fig. 2 shows only selected samples with the controls). Southern blot hybridisation with digoxigenin-labelled *L. intracellularis* NCTC 12657 verified that the amplified bands were composed of *L. intracellularis* specific DNA.

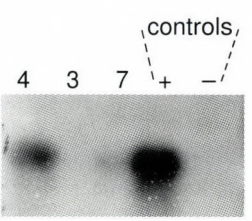

Fig. 2. Southern hybridisation of *L. intracellularis* DNA. Lanes 1–3: hybridisation pattern of PCR-amplified DNA from extracted faecal samples (4, 3 and 7, as shown previously in Fig. 1). Positivity of samples 4 and 7 was confirmed. Lanes 4–5: positive and negative controls, respectively

Although statistically valid sampling was done in one farm only, and our present study was not suitable for estimating the prevalence of *L. intracellularis* infection on herd or country level, the presence of the pathogen is clearly demonstrated by our findings. Further characterisation of the amplified DNA sequences and their comparison to the type strain are currently being performed in our laboratories.

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EFFECT OF METHIONINE AND ITS DERIVATIVES ON THE WEIGHT GAIN AND PROTEIN UTILISATION OF GROWING RATS

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Methionine and its derivatives are used as feed supplements. The objective of this study was to compare the effectiveness of DL-methionine (MET), DL-methionine-hydroxy-analogue (2-hydroxy-4-methylthio-butyric acid, MHA), and DL-S-methyl-methionine-sulphonium-chloride (SMM) in rats using low- (10 g/100g, BD10), medium- (15 g/100g, BD15) and high-protein (20 g/100g, BD20) diets. In a 10-day growth assay with weaning male rats (Wistar, SPF, LATI, Gödöllő, Hungary, 6 rats/group) methionine-deficient basic diets (BD10, BD15, BD20) were supplemented with 0, 1.5 and 3.0 g/kg MET, MHA or SMM. Extracted soybean meal was used as sole protein source. A protein-free diet was used for determining net protein ratio (NPR) indices. The weight gain of rats fed test diets fortified with 1.5 and 3.0 g/kg MET, MHA or SMM was significantly higher ($p < 0.05$) than that obtained with the unfortified diets. The NPR index of the diets was increased by MET, MHA or SMM supplementation, which increase was significant ($p < 0.05$) at 3.0 g/kg fortification level. However, there were no significant differences between the efficiencies of methionine derivatives on the same dietary protein levels. The weight gains of the rats were higher and the NPR indices of the diets were lower at a higher dietary protein level.

Key words: Methionine, derivatives, methionine-hydroxy-analogue, S-methyl-methionine, protein utilisation, rats

Methionine (MET) is an essential amino acid which is involved in protein biosynthesis as well as in various metabolic pathways (e.g. transmethylation, transsulphuration). The alpha-hydroxy analogue of MET (2-hydroxy-4-methylthio-butyric-acid, methionine-hydroxy-analogue, MHA) has been proved to be an effective replacement of MET in birds and in mammals (e.g. Reifsnyder et al., 1984; Friedman and Gumbman, 1989; Hegedűs et al., 1992). The S-methyl derivative of MET (S-methyl-methionine, SMM, also known as vitamin U) was shown to be not only a potent MET source but also an effective means of preventing oesophageal ulcer in pigs (Hegedűs et al., 1983).

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MET and its derivatives are used as feed supplements. The reason for this is the higher requirement for MET of the high-producing animals than the MET content of the usual feed ingredients. Originally crystalline DL-MET was used but in recent years, as a consequence of nutritional research, a less expensive MET-source, the liquid DL-MHA free acid has been introduced (Alimet™). However, the literature is not consistent concerning the relative efficiency of methionine derivatives when used in different feed formulas.

The objective of this study was to compare the effectiveness of MET, MHA and SMM in rat growth assay using diets with low, medium and high protein levels.

Materials and methods

The effect of MET (DL-methionine, Merck), MHA (DL-methionine-hydroxy-analogue free acid, Novus), and SMM (DL-S-methyl-methionine-sulphonium-chloride, Reanal) on weight gain and protein utilisation (NPR, net protein ratio) was tested in a 10-day growth assay with weaning male rats (WISTAR, SPF, LATI, Gödöllő, Hungary). Methionine-deficient basic diets having three different dietary crude protein levels (10, 15, 20 g/100g) (BD10, BD15, BD20) were supplemented with 0, 1.5 and 3.0 g/kg MET, MHA or SMM according to the active substance content of the products. Extracted soybean meal was used as sole protein source.

Rats were housed in wire cages. Feed and water were provided *ad libitum*. The temperature of the animal room was 28 °C and relative humidity was maintained between 60–80 %. Animals were assigned so that all treatment groups had the same initial mean body weight. Six rats were used per treatment.

The composition of the *basic diet* (%, w/w) was: cooking fat (lard) 14.25, sunflower oil 0.75, raw potato starch 10.00, vitamin and mineral premix (Bábolna, Hungary) 5.00, extracted soybean meal to give 10.00 crude protein in the test diet, and wheat starch ad 100. The composition of vitamin and mineral premix was adequate to meet the requirements of the rat (NRC, 1978).

A *protein-free diet* was used for determining the NPR indices. The composition of the protein-free diet corresponded to that of the test diet but wheat starch was used instead of soybean meal.

The criteria indicating the performance of experimental rats fed the test diets were weight gain and NPR index. *NPR* was calculated as follows: closing weight of a test animal minus closing weight of the control animal fed protein-free diet per gram of protein consumed by the test animal.

For *statistical* processing of the data Microsoft Excel-5 software was used (standard deviations, Student's *t*-test, correlation coefficients).

Results and discussion

Low-protein diet

The starting and closing mean weights, feed intake and N-intake of the rats as well as calculated NPR indices of the diets are summarised in Tables 1, 2 and 3. The weight gain values are illustrated in Fig. 1, while the tendencies of NPR indices are shown in Fig. 2.

Table 1

Effect of methionine sources on weight gain and protein utilisation of rats using 10% dietary crude protein level

Diets	Mean starting weight (n = 6) (g ± SD)	Mean closing weight (n = 6) (g ± SD)	Feed intake (g/10 day)	Crude protein intake (g/10 day)	NPR (g/g)
Protein-free control	53.17 ± 1.24	46.33 ± 3.71	34.90	0.00	–
BD10	53.25 ± 2.11	72.58 ± 4.07	62.83	6.28	4.18^{a} ± 0.33
BD10 + 1.5 g MET/kg	53.17 ± 1.60	80.33 ± 4.61	73.23	7.32	4.64^{b} ± 0.26
BD10 + 3.0 g MET/kg	53.20 ± 1.22	82.50 ± 5.15	78.66	7.87	4.60^{b} ± 0.29
BD10 + 1.5 g MHA/kg	53.25 ± 1.16	80.17 ± 4.11	72.30	7.23	4.68^{b} ± 0.24
BD10 + 3.0 g MHA/kg	53.17 ± 2.08	82.38 ± 5.25	76.56	7.66	4.71^{b} ± 0.49
BD10 + 1.5 g SMM/kg	53.17 ± 1.33	81.75 ± 4.36	75.63	7.56	4.68^{b} ± 0.25
BD10 + 3.0 g SMM/kg	53.08 ± 1.46	82.78 ± 5.64	79.59	7.96	4.58^{b} ± 0.31

DB10 = basic diet with 10% crude protein level; MET = DL-methionine; MHA = DL-methionine-hydroxy-analogue; SMM = DL-S-methyl-methionine; NPR = net protein ratio, values with different superscripts differ significantly ($p < 0.05$)

Table 2

Effect of methionine sources on weight gain and protein utilisation of rats using 15% dietary crude protein level

Diets	Mean starting weight (n = 6) (g ± sd)	Mean closing weight (n = 6) (g ± sd)	Feed intake (g/10 day)	Crude protein intake (g/10 day)	NPR (g/g)
BD15	53.17 ± 1.55	82.50 ± 8.64	74.99	11.25	3.48^{a} ± 0.36
BD15 + 1.5 g MET/kg	53.08 ± 1.38	101.07 ± 11.14	82.71	12.41	4.41^{b} ± 0.48
BD15 + 3.0 g MET/kg	53.17 ± 2.23	104.17 ± 10.31	85.75	12.86	4.50^{b} ± 0.45
BD15 + 1.5 g MHA/kg	53.00 ± 2.20	100.08 ± 9.71	80.37	12.06	4.46^{b} ± 0.43
BD15 + 3.0 g MHA/kg	53.00 ± 1.88	104.08 ± 11.43	83.81	12.57	4.59^{b} ± 0.50
BD15 + 1.5 g SMM/kg	53.92 ± 1.78	102.58 ± 8.05	85.45	12.82	4.39^{b} ± 0.34
BD15 + 3.0 g SMM/kg	53.00 ± 1.98	104.33 ± 10.78	84.60	12.69	4.57^{b} ± 0.47

BD15 = basic diet with 15% crude protein level; MET = DL-methionine; MHA = DL-methionine-hydroxy-analogue; SMM = DL-S-methyl-methionine; NPR = net protein ratio, values with different superscripts differ significantly ($p < 0.05$)

Table 3

Effect of methionine sources on weight gain and protein utilisation of rats using 20% dietary crude protein level

Diets	Mean starting weight (n = 6) (g ± sd)	Mean closing weight (n = 6) (g ± sd)	Feed intake (g/10 day)	Crude protein intake (g/10 day)	NPR (g/g)
BD20	53.00 ± 1.66	96.83 ± 9.93	83.04	16.61	3.04^{a} ± 0.32
BD20 + 1.5 g MET/kg	53.08 ± 1.97	105.00 ± 4.80	88.58	17.72	3.31^{a} ± 0.15
BD20 + 3.0 g MET/kg	53.00 ± 2.12	113.25 ± 8.64	92.03	18.41	3.64^{b} ± 0.28
BD20 + 1.5 g MHA/kg	53.08 ± 2.00	104.90 ± 6.36	85.79	11.76	3.41^{b} ± 0.21
BD20 + 3.0 g MHA/kg	53.00 ± 1.88	114.00 ± 11.36	85.30	17.06	3.97^{b} ± 0.30
BD20 + 1.5 g SMM/kg	53.08 ± 2.18	103.92 ± 8.99	86.14	17.23	3.34^{a} ± 0.29
BD20 + 3.0 g SMM/kg	53.08 ± 1.76	113.17 ± 9.25	87.83	17.57	3.81^{b} ± 0.31

BD20 = basic diet with 20% crude protein level; MET = DL-methionine; MHA = DL-methionine-hydroxy-analogue; SMM = DL-S-methyl-methionine; NPR = net protein ratio, values with different superscripts differ significantly ($p < 0.05$) from the unsupplemented diet (BD20)

The mean weight gain of rats fed the basic diet of 10 g/100 g dietary protein level (BD10) was 19.33 g, which increased to 27.16 and 29.30 g when 1.5 and 3.0 g/kg MET supplementation was used, respectively. These values were significantly higher ($p < 0.05$) as compared to those obtained with the BD10. The increase in weight gain between the lower and higher MET fortification level, however, was only tendency-like and not significant.

The mean weight gains of rats, as a result of 1.5 and 3.0 g/kg MHA supplementation of the BD10 diet, were 26.92 and 29.21 g respectively. These values were significantly higher ($p < 0.05$) than the weight gain on the unfortified control diet. However the increase in weight gain between 1.5 and 3.0 g/kg MHA fortification was not significant ($p > 0.05$).

The highest increase in weight gain was obtained by the SMM fortification (1.5 and 3.0 g/kg) of the BD10 diet (28.58 and 29.70 g respectively). The differences were significant ($p < 0.05$) as compared with the BD10 diet, however the higher weight gain on the higher fortification level was only a tendency and not significant ($p > 0.05$).

There were no significant differences between the effects of different methionine sources on the weight gain. However, at a lower fortification level MHA exerted a slightly lower activity than MET or SMM. On a higher fortification level the effect of MHA was equal to that of MET or SMM. These results correspond to those of our previous experiments (Hegedűs et al., 1992).

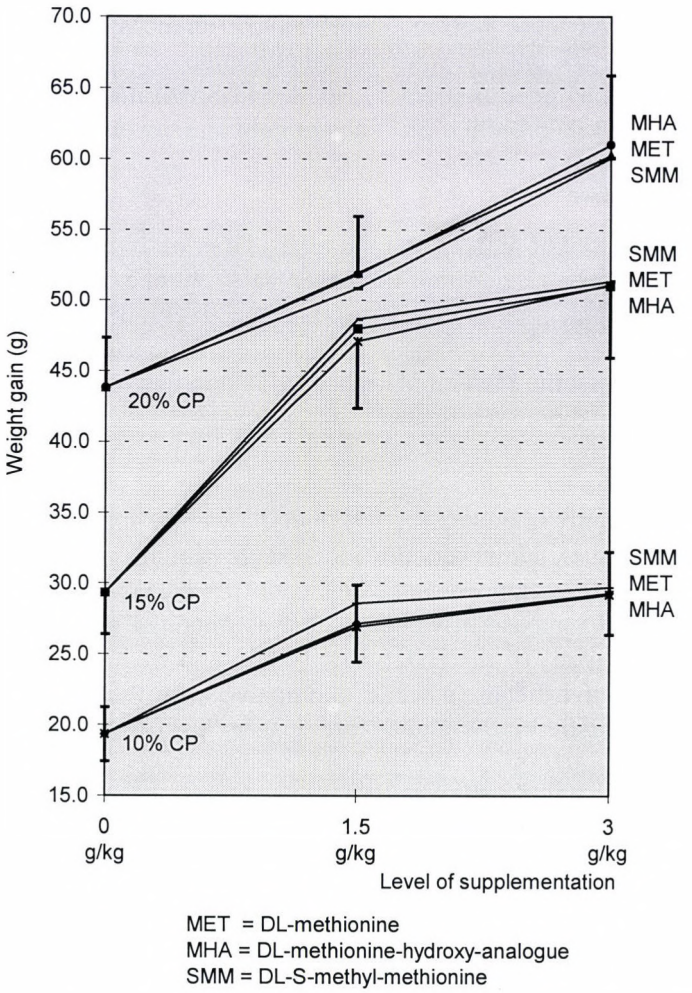

Fig. 1. Effect of supplementary methionine sources on the weight gain of rats fed different dietary protein levels

The mean nitrogen intake on the BD10 diet (1.01 g/10 days) slightly increased as a consequence of methionine source supplementation, however there were no positive correlations between feed intake and NPR values of the supplemented diets ($r = -0.59$).

The NPR values increased from 4.18 g/g (BD10) to 4.64 and 4.60 g/g (1.5 and 3.0 g/kg MET), 4.68 and 4.71 g/g (1.5 and 3.0 g/kg MHA), 5.09 and 4.58 g/g (1.5 and 3.0 g/kg SMM), respectively. The fortification of the BD10 diet with MET, MHA or SMM significantly improved the NPR values of the BD10 diet

($p < 0.05$), however there were no significant differences between the effect of the fortification levels and the sources of methionine.

It can be concluded that MET, MHA and SMM may be equally efficient in supplementing low-protein diets if adequately high levels are used.

Medium-protein diet

The mean weight gain of rats fed a basic diet of 15 g/100g dietary protein level (BD15) was 29.33 g, which increased to 47.99 and 51.00 g when 1.5 and 3.0 g/kg MET supplementation was used, respectively. These values were significantly higher ($p < 0.05$) compared to those of the BD15. The increase in weight gain between the lower and higher MET fortification level, however, was only a tendency, and not significant.

The mean weight gains of rats, as a result of 1.5 and 3.0 g/kg MHA supplementation of the BD15 diet, were 47.08 and 51.08 g, respectively. These values were significantly higher ($p < 0.05$) than the weight gain achieved on the unfortified control diet. However, the increase in weight gain between 1.5 and 3.0 g/kg MHA fortification was not significant ($p > 0.05$).

The highest increase in weight gain was obtained by the SMM fortification (1.5 and 3.0 g/kg) of the BD15 diet (48.66 and 51.33 g respectively). The differences were significant ($p < 0.05$) compared with the BD15 diet, however the higher weight gain on the higher fortification level was only a tendency and not significant ($p > 0.05$).

There were no significant differences between the effects of different methionine sources on the weight gain, however at the lower fortification level MHA exerted a slightly lower activity than MET or SMM. At the higher fortification level the effect of MHA was equal to that of MET or SMM.

The N-intake on the supplemented BD15 diets did not differ significantly. The correlation coefficient between N-intake of rats and NPR values of supplemented BD15 diets was 0.1 ($p > 0.05$).

The NPR values increased from 3.48 g/g (BD15) to 4.41 and 4.50 g/g (1.5 and 3.0 g/kg MET), 4.46 and 4.59 g/g (1.5 and 3.0 g/kg MHA), 4.39 and 4.57 g/g (1.5 and 3.0 g/kg SMM), respectively. The fortification of the BD15 diet with MET, MHA or SMM improved the NPR values of the BD15 diet significantly ($p < 0.05$), however there were no significant differences between the effect of the fortification levels and the sources of methionine.

It can be concluded that MET, MHA and SMM may be equally efficient in supplementing medium-protein diets if adequately high levels are used.

High-protein diet

The mean weight gain of the rats fed the basic diet of 20 g/100g dietary protein level (BD20) was 43.83 g, which increased to 51.92 and 60.25 g when 1.5 and 3.0 g/kg MET supplementation was used, respectively. These values were significantly higher ($p < 0.05$) compared to that of the BD20. The increase in weight gain between the lower and higher MET fortification level was also significant ($p < 0.05$).

The mean weight gains of rats, as a result of 1.5 and 3.0 g/kg MHA supplementation of the BD20 diet, were 51.82 and 61.00 g, respectively. These values were significantly higher ($p < 0.05$) than the weight gain on the unfortified control diet. The increase in weight gain between 1.5 and 3.0 g/kg MHA fortification was also significant ($p < 0.05$).

SMM fortification (1.5 and 3.0 g/kg) of the BD20 diet increased the weight gain of rats significantly (50.84 and 60.09 g, respectively). The difference between the lower and higher fortification levels was also significant ($p < 0.05$).

There were no significant differences between the effects of different methionine sources (MET, MHA, SMM) on the weight gain on the same fortification levels (51.92, 51.82, 50.84 on 1.5 g/kg, 60.25, 61.00, 60,09 on 3.0 g/kg respectively).

Feed and N-intake values did not differ significantly on supplemented BD20 diets. The correlation coefficient between N-intake of rats and NPR values of supplemented BD20 diets was –0.06 ($p > 0.05$).

The *NPR values* increased from 3.04 g/g (BD20) to 3.31 and 3.64 g/g (1.5 and 3.0 g/kg MET), 3.41 and 3.97 g/g (1.5 and 3.0 g/kg MHA), 3.34 and 3.81 g/g (1.5 and 3.0 g/kg SMM), respectively.

The fortification of the BD20 diet with MET, MHA or SMM increased the NPR values significantly ($p < 0.05$) on the 3.0 g/kg fortification level; however, on the 1.5 g/kg level only MHA increased the NPR value of the BD20 diet significantly. The higher fortification levels were more effective than the lower ones. The effect of MHA was slightly higher than that of SMM and MET (Fig. 2).

It can be concluded that MET, MHA and SMM are efficient in supplementing high-protein diets. The effect of MHA on the NPR value of the BD20 diet was slightly superior to that of SMM or MET. Using the high-protein diet (BD20) the differences in NPR values were higher as a consequence of MET, MHA and SMM supplementation than in the case of medium- (BD15) or low- (BD10) protein diets. However these differences were only tendencies and statistically not significant ($p > 0.05$).

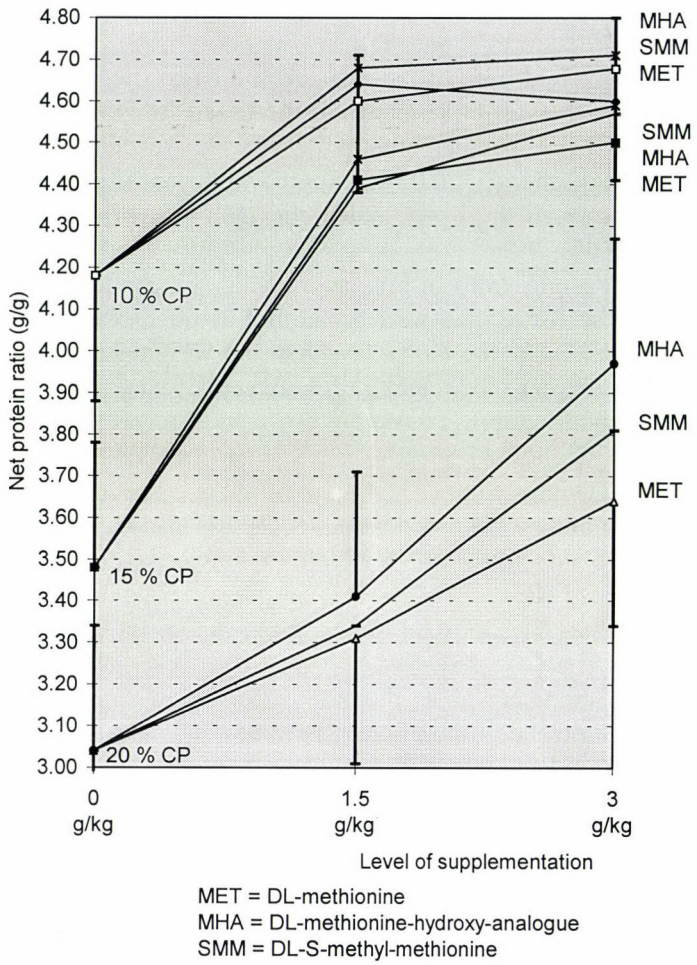

Fig. 2. Effect of supplementary methionine sources on protein utilisation of rats fed different dietary protein levels

The effect of dietary protein level

The effects of dietary protein levels on the weight gain of rats and NPR values of diets are illustrated in Figs 1 and 2. The weight gain of rats was higher on higher protein levels, however there were only small differences on the same protein level as a consequence of supplementation of the diets with different methionine sources (MET, MHA, SMM) on the same fortification level.

The NPR values of diets were lower as a consequence of the higher protein level. There were only small differences on the same protein level as a consequence of supplementation of the diets with different methionine sources

(MET, MHA, SMM) on the same fortification level. However, there was a tendency on high protein level for a greater deviation between the effects of the different methionine sources.

MHA proved to be slightly more effective on the higher fortification level (3.0 g/kg) using higher dietary protein level (20 g/100g).

The slight differences between the effects of MET, MHA and SMM can be explained by their chemical and biochemical properties. MHA as a strong organic acid may have beneficial dietetic effects as well if high-protein diets are used. MHA is absorbed by passive diffusion, and its toxicity is lower on high levels. The acidifying effect of MHA may also be beneficial if using high-protein diets with high buffer capacity. However, MHA must be transaminated to form MET (Lewis and Baker, 1995) which may explain its slightly lower efficiency on lower supplementation levels using low protein diets.

The slightly higher efficiency of SMM compared to MET can be explained by its additional methyl group. Thus SMM will supply a MET molecule if it is involved in transmethylation reactions.

The differences observed in this experiment were small and allow us to draw the overall conclusion that MET, MHA and SMM can be equally effective in supplementing methionine-deficient diets if adequately high levels are used.

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DETECTION OF ANTIBODIES TO *NEOSPORA CANINUM* IN BOVINE POSTABORTION BLOOD SAMPLES FROM HUNGARY

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In order to obtain data on the occurrence of the protozoan parasite *Neospora caninum* as a potential factor in the aetiology of reproduction problems in cattle, 97 postabortion sera were examined by ISCOM ELISA and IFAT for the presence of antibodies to *N. caninum*. The results showed 10% and 9% seropositivity by the ELISA and IFAT, respectively. In 2 of the 10 seropositive cases no other fetopathogenic agents (such as *Chlamydia* sp., *Leptospira* spp. or bovine viral diarrhoea virus) were detected. These data confirm the presence of *N. caninum* in cattle in Hungary.

Key words: *Neospora caninum*, abortion, cattle, ISCOM ELISA, IFAT

Neospora caninum is an apicomplexan protozoan parasite, which, until defined as a distinct species in 1988, was misdiagnosed to be *Toxoplasma gondii* (Dubey et al., 1988). Its life cycle is not completely known, since only tachyzoites (quickly multiplying stages in different host cells) and tissue cysts (located in muscular tissues and the nervous system) have been identified. A carnivorous definitive host is suspected, however, oocysts are not shed by infected dogs, cats and other carnivores studied so far (Dubey and Lindsay, 1996). Natural *N. caninum* infection has been found in several mammalian species including dogs, cattle, sheep, goats, and horses. The clinically most important disease entities are ascending paralysis in young dogs (Dubey et al., 1988) and abortion in cattle (Dubey and Lindsay, 1996).

N. caninum appears to have a world-wide distribution and has been found in a dog also in Hungary (Sréter et al., 1992), however, its presence in cattle has not previously been investigated in this country. In our study, 97 serum samples of aborted cows from Hungary were analysed by IFAT (immunofluorescent antibody test) and ELISA (enzyme-linked immunosorbent assay),

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the latter utilising the specificity of ISCOM (immunostimulating complex) antigen (Björkman et al., 1997) prepared from membrane fractions of *N. caninum* tachyzoites.

Materials and methods

Serum samples

Blood samples were taken from dairy cows with a recent history of abortion. The animals originated from different locations in Hungary. Thirty-nine of the samples were submitted from different counties directly to the Central Veterinary Institute, Budapest, 42 from Hajdú-Bihar and 16 from Borsod-Abaúj-Zemplén County. The blood samples were centrifuged at 1000 *g* for 5 min and the sera collected and stored at –20 °C. Routine serological diagnostic tests were performed to detect antibodies to other potential causes of abortion, such as *Chlamydia* sp. (complement fixation test), *Leptospira* spp. (agglutination test) and bovine virus diarrhoea virus (BVDV; ELISA).

ELISA

The ELISA was performed according to Björkman et al. (1997). Briefly, detergent extracted *N. caninum* tachyzoite antigens were incorporated into ISCOMs which were used to coat microtitre plates. Free binding sites were blocked with 10% *N. caninum* antibody free horse serum diluted in phosphate buffered saline, pH 7.4, containing 0.05% Tween 20 (PBST). Test sera and control sera from infected as well as non-infected cattle were diluted 1:100 in PBST and analysed in duplicates. Horseradish peroxidase conjugated mouse monoclonal antibodies to bovine IgG1 (Svanova, Uppsala, Sweden) were used as secondary antibody and 3, 5, 3', 5'-tetramethylbenzidine and 0.006% H_2O_2 as substrate. The reaction was stopped by adding 10% sulphuric acid and the absorbances were read at 450 nm. Absorbance values were adjusted by dividing the mean values of the duplicates by that of positive controls. The cut-off value was 0.20.

IFAT

The IFAT was performed according to Björkman et al. (1997). Briefly, purified *N. caninum* tachyzoites grown in cell culture in RPMI medium containing 5% *N. caninum* antibody free horse serum were dried onto 18-well microscope slides as antigen. The sera were tested in 2-fold serial dilutions from 1:320 to 1:2560 in PBS. Sera of infected and non-infected animals were included as controls in each series of analysis. Fluorescein isothiocyanate labelled rabbit-anti-bovine IgG (Sigma Chemical Company, St. Louis, USA)

was used as conjugate at a dilution of 1:200 in PBS. Samples showing unbroken tachyzoite membrane fluorescence at the dilution 1:640 or higher were regarded as positive.

Results

In the *Neospora* ISCOM ELISA 10 (10%) out of 97 sera had absorbance values exceeding the cut-off of 0.2 absorbance units. Nine of these samples were also positive by the IFAT, giving an overall seropositivity rate of 9%. As shown in Table 1, seropositive animals were found in different parts of the country. Antibodies to *Chlamydia* sp. were detected in 2, to *Leptospira* spp. in 3, and to BVDV in 5 of the *N. caninum* positive samples (Table 1). In 2 animals only antibodies to *N. caninum* were detected. One cow was found seropositive to all abortifacient agents investigated. Antibodies to *N. caninum* were not detected in any of the 16 samples submitted from Borsod-Abaúj-Zemplén County.

Table 1

Serological data of samples from aborted cows that were found seropositive to *Neospora caninum* (Nc)

Geographical location	Serology				
	Nc ELISA abs.	Nc IFAT titre	BVDV	*Chlamydia*	*Leptospira*
Nagykőrös (Pest)	0.776	640	+	–	–
Székszend (Komárom)	0.395	320	–	–	–
Nyírbátor*	0.703	640	+	+	+
Nyírbátor*	0.734	1280	+	–	–
Dombrád	1.047	640	+	–	–
Hajdúböszörmény	0.527	640	–	+	–
Mezőhék	0.646	640	–	–	+
Magyarhomorog**	0.723	640	–	–	–
Magyarhomorog**	0.894	1280	–	–	+
Balmazújváros	0.835	1280	+	–	–

* and ** from the same herd

Discussion

The serological tests used in this survey are suitable for the diagnosis of *N. caninum* infections in cattle (Dubey et al., 1997). When compared with the IFAT the ISCOM ELISA showed a 100% sensitivity and 96% specificity (Björkman et al., 1997). These figures indicate that although antibodies are demonstrated by the ISCOM ELISA, sera from some infected animals may remain negative by the

IFAT, as observed in the present study. This is likely to be attributable to different antigens being used in the two techniques.

Earlier published seroepidemiological studies indicated geographic differences in the occurrence of neosporosis. In parts of the USA, 19% of abortions in dairy cows was attributed to neosporosis (Anderson et al., 1991). In Scotland, 9% seropositivity was found among aborted cows (Trees et al., 1994), and in Northern Ireland, 13% of postabortion sera were positive by IFAT (McNamee et al., 1996). Samples collected at German dairy farms with abortion and reproduction problems showed 4% seropositivity (Conraths et al., 1996). Neosporosis as a potential cause of bovine abortion was also identified in other European countries, like Sweden (Holmdahl et al., 1995; Björkman et al., 1996), Denmark (Agerholm and Barr, 1994), The Netherlands (Wouda et al., 1995), Switzerland (Gottstein et al., 1997) and Spain (Quintanilla-Gozalo et al., 1997). However, no seropositivity was found by the analysis of 37 sera from aborted cows in the Czech Republic (Bretislav Koudela, personal communication).

The prevalence of antibodies to *N. caninum* was found to be higher in cows with a history of abortion than in those without such problems (Trees et al., 1994; McNamee et al., 1996). Most chronically infected cows give birth to infected calves, and the infection may be present without clinical manifestations (Paré et al., 1996). Since the only known way of transmission is via the placenta and the infection can be transmitted during consecutive pregnancies (Dubey and Lindsay, 1996), it has been suggested that cows with a history of neosporosis should not be used for further breeding (Thurmond and Hietala, 1995).

The presence of antibodies to *N. caninum* in an aborted cow indicates infection with the parasite, but this cannot always be used as a basis for the conclusion that *N. caninum* in fact caused the abortion (Dubey et al., 1997). In the present study, most of the *N. caninum* seropositive cows also carried antibodies to other abortifacient agents, e.g. *Leptospira* spp., *Chlamydia* sp. and/or BVDV. Therefore, it cannot be excluded that factors other than *N. caninum* may have contributed to the outcome of the pregnancy in these cows. Nevertheless, it has been estimated that in *N. caninum* infected cows the risk of abortion is twice as high as in noninfected ones, and that half of the abortions occurring in infected cows may be attributed to neosporosis (Paré et al., 1997). Further investigations of herds with abortion problems in Hungary will be necessary before establishing the role of neosporosis as a cause of bovine abortion in this country.

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STUDIES ON THE OCCURRENCE OF ACTINOSPOREAN STAGES OF MYXOSPOREANS IN LAKE BALATON, HUNGARY, WITH THE DESCRIPTION OF TRIACTINOMYXON, RAABEIA AND AURANTIACTINOMYXON TYPES

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In the framework of a one-year survey, the actinosporean infection of the oligochaete fauna living in the mud and on the vegetation of Lake Balaton, Hungary was studied. A total of 10 types of actinospores were isolated from the oligochaetes *Tubifex tubifex* and *Limnodrilus hoffmeisteri* collected during the survey, which could be classified into the triactinomyxon, raabeia and aurantiactinomyxon groups. The drawings depicting the individual actinospore types are presented on a plate and their characteristic dimensions summarised in tables. The prevalence and seasonality of actinosporean infections observed in the two oligochaete species are illustrated graphically. Infection by actinospores showed pronounced seasonality. In the spring and summer the prevalence of triactinomyxon infection in *T. tubifex* exceeded 30 and 40%, respectively, while in the autumn it dropped to 6%. In *T. tubifex* raabeia infection of 3% prevalence was detected only in the summer period. In *L. hoffmeisteri* the peak of aurantiactinomyxon, raabeia and triactinomyxon infection occurred in the summer with a prevalence of 21, 10 and 8%, respectively. Actinosporean infection in the individual Oligochaeta species showed higher prevalence values than had been reported in the literature, which fact may be explained by the novelty of the examination technique used. Four types of actinospores found in Lake Balaton are identical with the actinospores already described. It cannot be determined with absolute certainty which stages of myxosporeans parasitic in Lake Balaton fishes are identical with the 10 actinospores described.

Key words: Actinospore, myxosporeans, Myxozoa, survey, Lake Balaton, triactinomyxon, raabeia, aurantiactinomyxon

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The first report on actinosporeans was published by Stolc (1899), who described these organisms found in Bohemia as parasites related to myxosporeans. Although nearly one hundred years have elapsed since their first description, until quite recently only few researchers had studied actinosporeans. Of them, Ikeda (1912) and Mackinnon and Adam (1924) detected tetractinomyxon and triactinomyxon forms in England, while in Poland Janiszewska (1955, 1957) performed detailed studies on the morphology, ecology and systematics of actinosporeans. The ultrastructure and biology of these parasites have been studied most intensively by Marques (1984), Lom and Dykova (1997), and Lom et al. (1997a). Research on actinosporeans gathered momentum after Wolf and Markiw (1984) had demonstrated that actinosporeans did not constitute an independent taxonomic unit but corresponded to fish-parasitic myxosporean stages developing in oligochaetes. After Wolf and Markiw (1984), other researchers also produced experimental evidence that in the developmental cycle of a given species of Myxosporea intrapiscine myxosporean and intra-oligochaete (El-Matbouli and Hoffmann, 1989; Ruidisch et al., 1991; El-Matbouli et al., 1992; Grossheider and Körting, 1992; El-Matbouli and Hoffmann, 1993; Kent et al., 1993; Yokoyama et al., 1993*a*; El-Matbouli et al., 1995; Kent et al., 1995; Uspenskaya, 1995; Yokoyama et al., 1995; Trouillier et al., 1996; Yokoyama, 1997; El-Mansy and Molnár, 1997*a*, 1997*b*) or intra-polychaete (Bartholomew et al., 1997) actinosporean stages alternated. Relying on these studies, Kent et al. (1995) proposed that the class Actinosporea should be merged into the class Myxosporea as a synonym of the latter, and that the names of actinosporean genera should in the future be used only for typing actinospores developing in oligochaetes. The major taxonomic changes of recent years are reflected in the works of Smothers et al. (1994), Kent et al. (1995), Siddall et al. (1995), and Schlegel et al. (1996), who reassigned phylum Myxozoa, earlier regarded by many authors as protozoa, from protozoan to metazoan parasites.

The actinosporean infection of oligochaetes in fish farms and natural waters in relation to the developmental cycle of myxosporeans has so far been studied among others by Hamilton and Canning (1987), Székely (1989), Burtle et al. (1991), Pote and Waterstrat (1993), Koller (1994), Pallós (1995), McGeorge et al. (1997), and El-Mansy et al. (1998*b*).

Myxosporean infections of fishes and intrapiscine myxosporeans are relatively well studied in Lake Balaton (Jaczó, 1940; Molnár and Székely, 1995; Székely and Molnár, 1996–1997), but no study has been done on actinosporean stages until now.

The present paper reports on a one-year survey made on the occurrence of actinospores of Myxosporea in oligochaete alternate hosts in Lake Balaton.

Materials and methods

The survey was carried out in Lake Balaton, Hungary, between March 1996 and March 1997. Water-weed and mud collected from the lake were transported to the laboratory for the isolation of oligochaetes and then actinospores. In the course of the one-year survey two oligochaete species, *Tubifex tubifex* (Müller) and *Limnodrilus hoffmeisteri* (Claparéde) [identified according to Brinkhurst (1963)] were examined for the occurrence of actinospores.

The methods used for the isolation of actinospores were the same as those described by Yokoyama et al. (1991) and El-Mansy et al. (1998*b*). Actinospores found in this survey were measured and identified as described by Lom et al. (1997*b*).

Results

From the 1164 specimens of the two oligochaete species studied (*T. tubifex* and *L. hoffmeisteri*) a total of 10 actinospore types (Figs 1, 2.1 and 2.2) were isolated from Lake Balaton. The actinospores found belonged to three main groups (triactinomyxon, raabeia and aurantiactinomyxon). Of the 807 *Tubifex* specimens examined, 267 (33%) were infected with triactinomyxon and 9 (1%) with raabeia spores (Fig. 3). Of the 357 *Limnodrilus* specimens, 10 (3%) proved to be infected with aurantiactinomyxon, 7 (2%) with triactinomyxon, and 5 (1.4%) with raabeia spores (Fig. 4).

Infection with actinospores exhibited a pronounced seasonality. In the spring and summer the prevalence of triactinomyxon infection in *T. tubifex* exceeded 30 to 40%, while in the autumn and winter it dropped to 6 and 0%, respectively. Raabeia infection in *T. tubifex* was only observed during the summer. At that time its prevalence went up to 3% (Fig. 5). In *L. hoffmeisteri* the highest prevalence of actinospore infection occurred in the summer when it reached 21% with aurantiactinomyxon, 10% with raabeia and 8% with triactinomyxon spores (Fig. 6). The main parameters of the actinospores found are presented in Tables 1–3.

Discussion

Owing to the increased interest taken in myxosporean diseases and the successful progress of experimental studies in recent years, actinosporean infections of oligochaetes or polychaetes have been investigated by numerous authors, among others by Mackinnon and Adam (1924), Markiw (1986), El-Matbouli and Hoffmann (1989), Yokoyama et al. (1991), El-Matbouli and

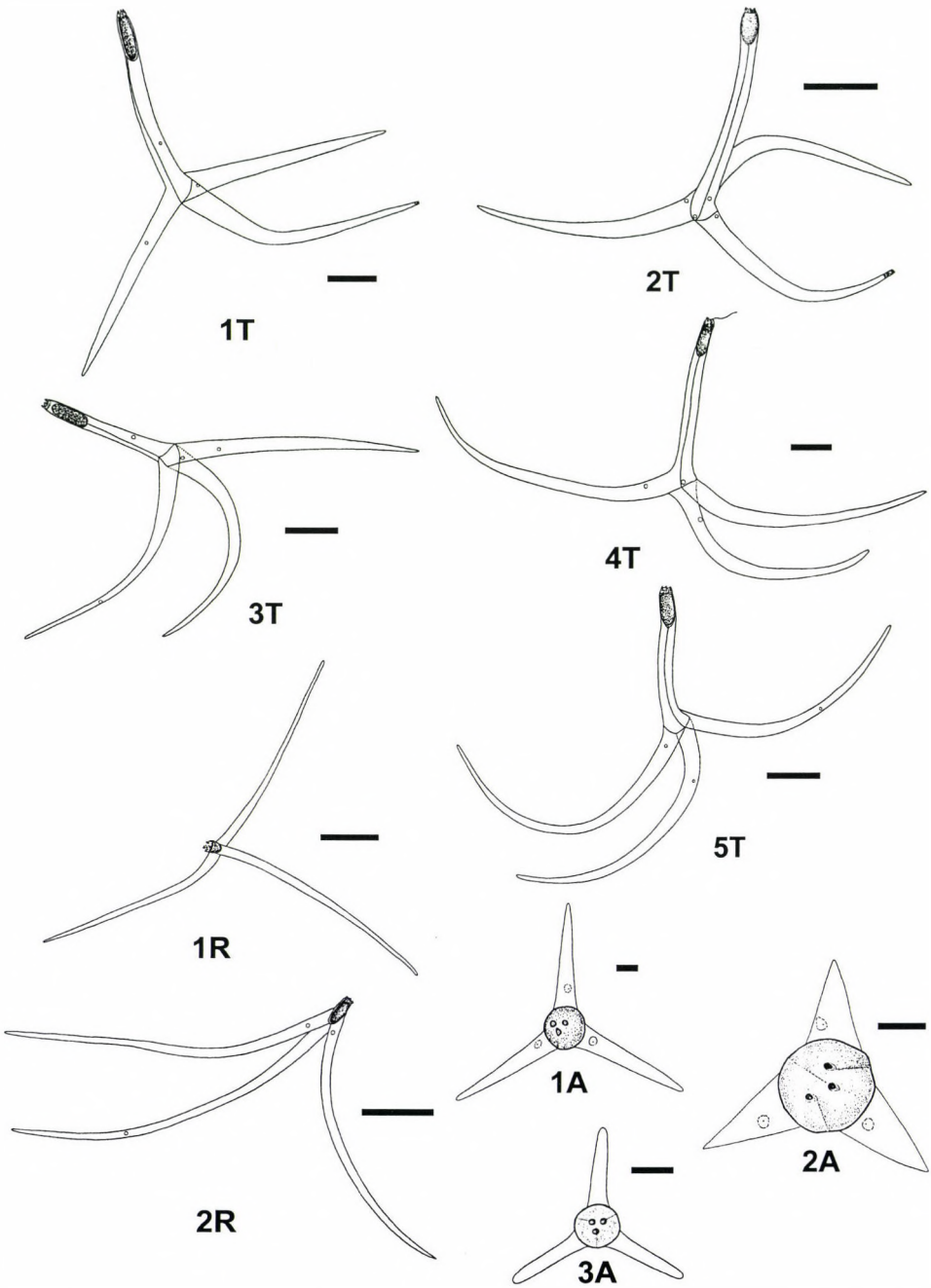

Fig. 1. Line drawings of actinospores found during a one-year survey of Lake Balaton oligochaetes. Triactinomyxon types: 1T, 2T, 3T, 4T and 5T, raabeia types: 1R and 2R, aurantiactinomyxon types: 1A, 2A and 3A. Bar = 50 μm

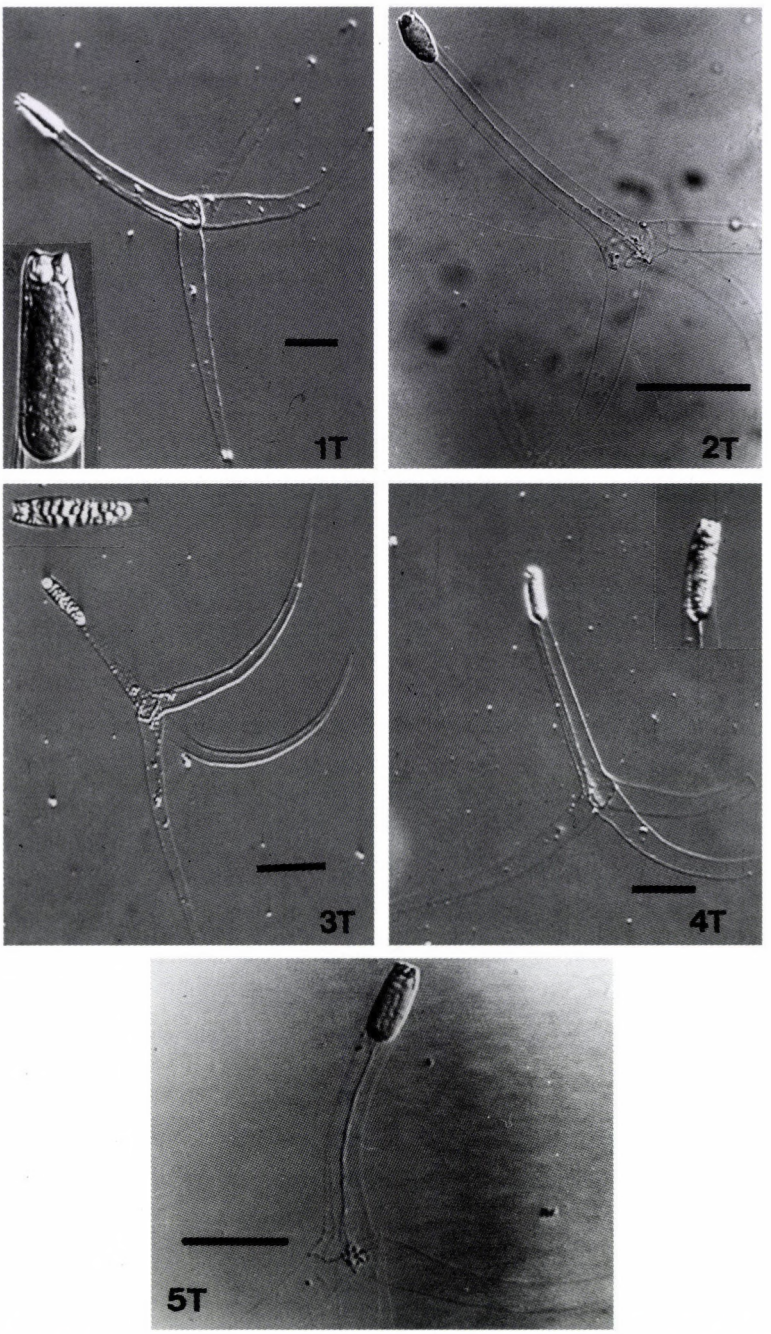

Fig. 2.1. Microphotographs of live preparations of triactinospores (types 1–5) found during a one-year survey of oligochaetes in Lake Balaton. Bar = 50 μm

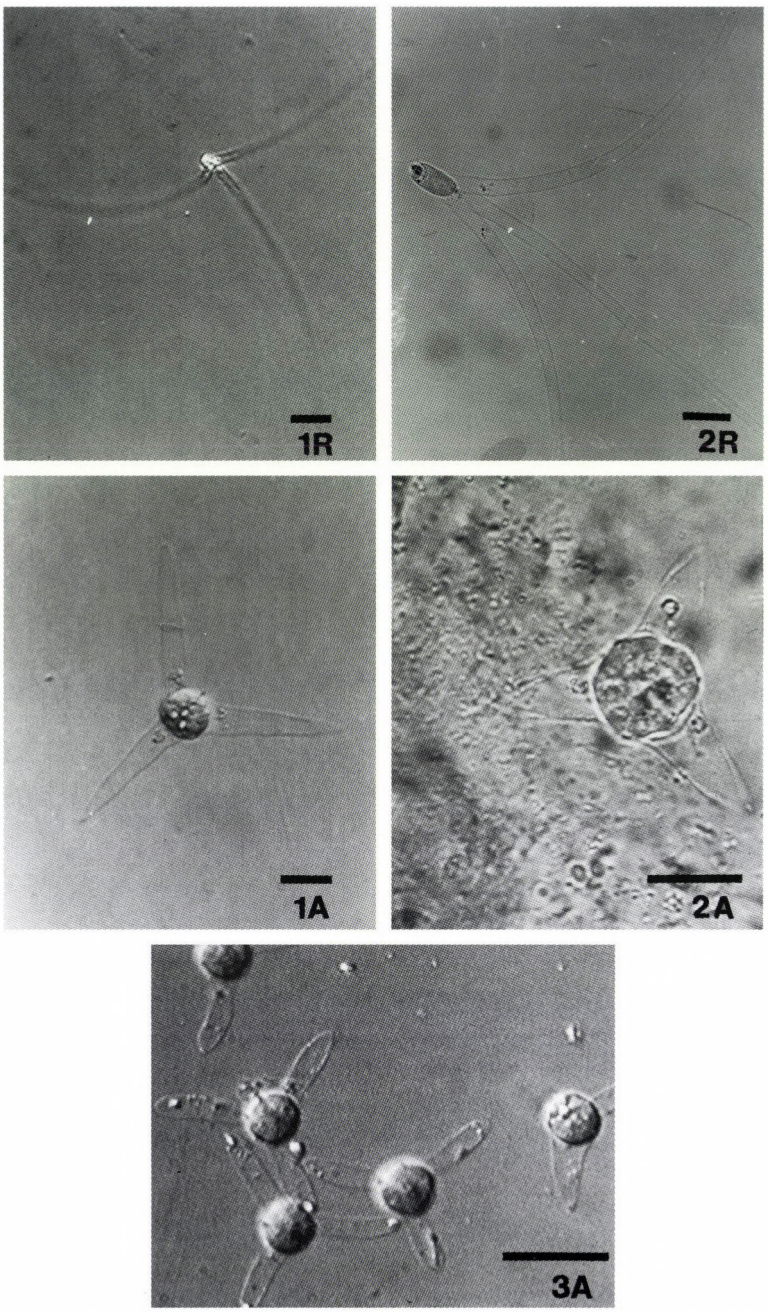

Fig. 2.2. Microphotographs of live preparations of raabeia spores (types 1–2) and aurantiactinomyxon spores (types 1–3) found during a one-year survey of oligochaetes in Lake Balaton. Bar = 20 μm

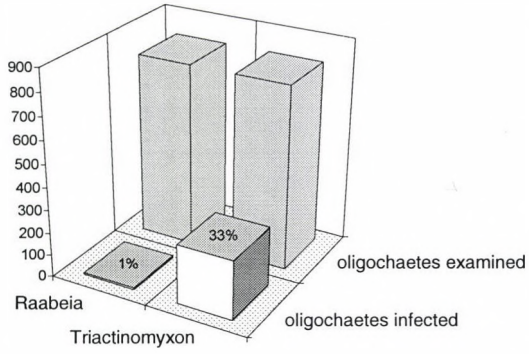

Fig. 3. Prevalence of infection in *Tubifex tubifex* (all year)

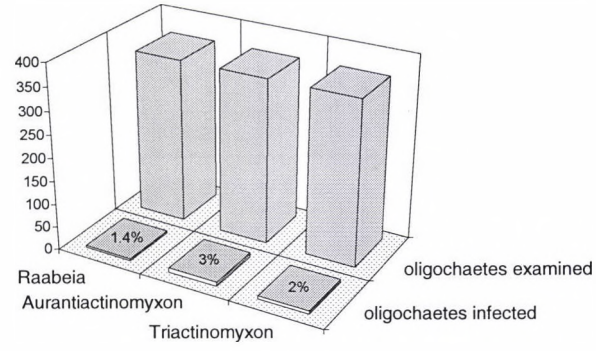

Fig. 4. Prevalence of infection in *Limnodrilus hoffmeisteri* (all year)

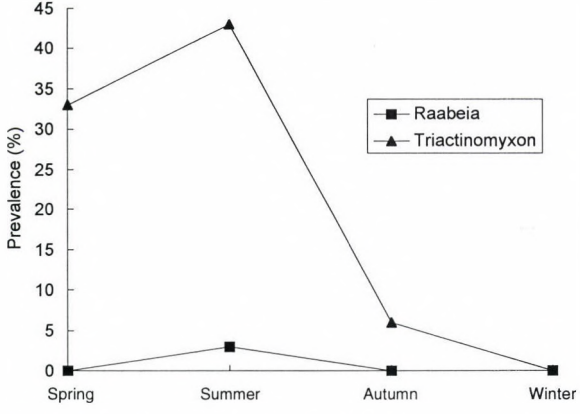

Fig. 5. Seasonality of actinosporean infection in *Tubifex tubifex*

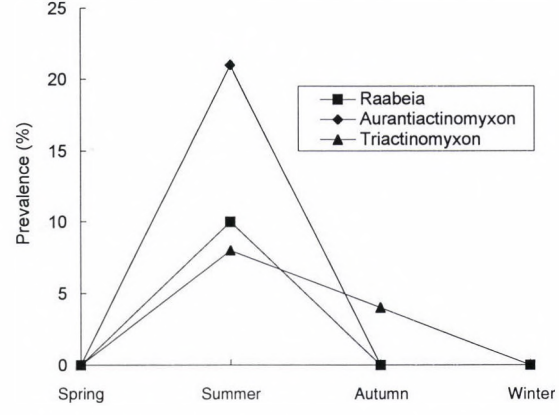

Fig. 6. Seasonality of actinosporean infection in *Limnodrilus hoffmeisteri*

All-year and seasonal prevalence of actinosporean infection of oligochaetes in Lake Balaton, Hungary from March 1996 to March 1997

Table 1

Average dimensions of triactinomyxon types found during the survey (in μm)

Triactino-myxon type no.	Origin of spores	Polar capsule dimensions	Spore body dimensions	Secondary cell dimensions	No. of secondary cells	Style length	Style width at start/ at end	Caudal process dimensions	Whole length
1	water (*Tubifex* or *Limnodrilus)*	7.1 × 3.5	50.6 × 12.9	ND	ND	123.6	21.2	230 × 18.8	404.1
2	water (*Tubifex* or *Limnodrilus)*	3.0 × 2.9	25.4 × 10.6	ND	ND	117.7	15.3/9.4	152.2 × 17.6	295.2
3	water (*Tubifex*)	4.7 × 3.5	44.7 × 11.8	3.5 × 3.5	21	87.1	20	224.6 × 17.5	360
4	water (*Limnodrilus*)	8.0 × 5.9	45 × 12.9	ND	ND	149	23.5/14.1	281.7 × 20.8	478.4
5	water (*Tubifex* or *Limnodrilus)*	5.9 × 3.5	37.7 × 13.5	ND	ND	90.6	12.9/17.7	249 × 16.1	377.8

ND = not determined

Table 2

Average dimensions of raabeia types found during the survey (in μm)

Raabeia type no.	Origin of spores	Polar capsule dimensions	Sporoplasm dimension	Spore body dimensions	Caudal process dimensions	Whole length (from polar capsules to the end of caudal process)
1	water (*Limnodrilus)*	5.9 × 4.7	8.2 × 12.4	14.1 × 12.4	202.8 × 8.2	216.9
2	*Tubifex*	5.7 × 4.0	16.0 × 6.8	21.7 × 7.7	209.4 × 6.6	231.1

Table 3

Average dimensions of aurantiactinomyxon types found during the survey (in μm)

Aurantiact. type no.	Origin of spores	Caudal process dimensions	Polar capsule dimension	Spore cavity diameter	Largest span
1	water (*Branchiura sowerbyi)*	51.3 × 9.5	2.3 × 2.3	18.8	103.2
2	*Limnodrilus*	22.6 × 11.7	2.8 × 2.0	21.1	52.2
3	water (*Branchiura sowerbyi)*	17.2 × 3.9	1.4 × 1.4	9.9	39.5

Hoffmann (1993), Kent et al. (1993), Yokoyama et al. (1993*a, b*), El-Matbouli et al. (1995), Kent et al. (1995), Uspenskaya (1995), Yokoyama et al. (1995), Trouillier et al. (1996), and Bartholomew et al. (1997). Most of the above-listed authors reported a relatively low (around 1%) prevalence of infection in oligochaete populations. Yokoyama et al. (1993*a, b*) were the only investigators who detected > 4% actinosporean infection in *Branchiura sowerbyi* in a goldfish-culturing pond. In a survey of the actinosporean infection of oligochaetes in natural waters of Hungary, Székely (1989) and Pallós (1995) also found similarly low levels of infection. In contrast, in the survey of El-Mansy et al. (1998*b*) and in the present study we obtained high prevalence values (e.g. triactinomyxon infection of *T. tubifex* showed a prevalence of 33–43% in certain periods of the year). These values are significantly higher than those reported earlier by other authors. The higher values recorded by us, however, do not mean that the level of actinospore infection is so much higher in Lake Balaton oligochaetes than in oligochaetes from other habitats. The observed difference can be attributed primarily to the different examination technique used by us (El-Mansy et al., 1998*b*). Today it is already well known that a given oligochaete can be infected with different actinosporean developmental stages for several months, while the mature actinospores will be excreted only after an about 3-month period of intra-oligochaete development. The fact that in this study individual oligochaete specimens were regularly examined over a 3-month period, greatly elevated the ratio of positive individuals and revealed that a single examination of a given worm cannot provide reliable data on the prevalence of actinosporean infection, as it shows exclusively the ratio of worms that release mature spores at the time of examination.

The data obtained by us indicate that actinospore infection of oligochaetes persists throughout the year and is characterised by seasonality manifesting itself primarily in the appearance of worms containing mature spores, which reaches its peak in the warmer months.

Although myxosporeans parasitise several lower ranked vertebrates, the great majority of species are known from fishes. Accordingly, the actinosporeans found in this survey should first of all be regarded as fish parasitic myxosporeans. It seems to be evident that a habitat harbouring several fish species must be rich in myxosporean species and evidently also in actinospores. At the present time about 41 fish species are known to live in Lake Balaton (Biró, 1995).

The fish fauna of cold waters is relatively deficient in species and, in a similar way, fish species (salmonids, goldfish and catfish) cultured in monocultures are infected only with a scarce number of myxosporean species (Pote and Waterstrat, 1993; Yokoyama et al., 1993*a, b*; McGeorge et al., 1997). In addition to our improved actinospore examination technique, this could also account for the higher prevalence of actinosporeans found by us in Lake Balaton oligochaetes.

The actinospores found during the present survey were defined on the basis of the old taxonomic terms (triactinomyxon, raabeia and aurantiactinomyxon) described by Janiszewska (1955, 1957) and Marques (1984); however, we agree with the view of El-Matbouli et al. (1992), Kent et al. (1993), Yokoyama et al. (1993*a*), Kent et al. (1995), Yokoyama et al. (1995), Trouillier et al. (1996), and McGeorge et al. (1997), i.e. that these cannot be regarded as independent taxonomic units. The accepted names are used only for denoting the type of developmental stage of a given myxosporean species. The number of the 10 actinospore types detected by us from Lake Balaton roughly corresponds to that of myxosporean species hitherto found in the lake. We do not venture to identify any of the detected actinospore types as a developmental stage of any of the myxosporean species occurring in the lake in question. However, based upon the experimentally proven developmental cycles reported in the literature (Wolf and Markiw, 1984; El-Matbouli and Hoffmann, 1989; El-Matbouli et al., 1992; Benajiba and Marques, 1993; El-Matbouli and Hoffmann, 1993; Kent et al., 1993; Uspenskaya, 1995; Yokoyama et al., 1995; El-Mansy and Molnár, 1997*a*, 1997*b*) it can be assumed that triactinomyxons and raabeias represent the developmental stages of species belonging to the *Myxobolus* genus, while aurantiactinomyxons may be the developmental stages of species included in the genera *Myxidium, Zschokkella, Hoferellus* or *Thelohanellus*.

Although a relatively large number of myxosporeans are known from Lake Balaton fishes (Molnár and Székely, 1995; Székely and Molnár, 1996–1997), their actinosporean stages have not been identified. Only three species (*Myxobolus drjagini*, *M. hungaricus* and *M. portucalensis*) can be regarded as exceptions, as their actinosporean stages have already been revealed in experimental studies (El-Mansy and Molnár, 1997*a*, 1997*b*; El-Mansy et al., 1998*a*). All of the three *Myxobolus* species developed in their *Tubifex* alternate hosts into triactinospores. In some dimensions (e.g. the length of the style and the caudal processes) these triactinospores resembled some triactinospores found during the survey but there were major differences in the shape of the spores and in the number of secondary cells in the sporoplasm. In a similar way, none of the 5 triactinospore types could be identified with triactinospores described by other authors (Hamilton and Canning, 1987; El-Matbouli and Hoffmann, 1993; McGeorge et al., 1997) who studied actinosporeans in waters inhabited by salmonid fishes. None of the triactinospores proved to be identical with the triactinospores found by us during a survey of actinosporeans of oligochaetes from a fish pond (El-Mansy et al., 1998*b*).

Both of the raabeia types found by us differed from the forms hitherto described (Janiszewska, 1957; Janiszewska and Krzton, 1973; Yokoyama et al., 1995; McGeorge et al., 1997). Although the raabeia types found by us in Lake Balaton oligochaetes have caudal processes similar in size to that described by

Yokoyama et al. (1995) from *Branchiura sowerbyi* and, moreover, type 2 even has spore body parameters identical with those of the latter, type 2 described by us has much larger polar capsules than that reported by the above-cited Japanese authors. On the other hand, both raabeia types found in our survey in Lake Balaton are identical with raabeia spores found by us during a survey of fish pond oligochaetes (types 1 and 2 from Lake Balaton are identical with types 2 and 4 from the fish ponds, respectively; El-Mansy et al., 1998*b*).

By their dimensions, all the 3 aurantiactinomyxon types differed from the forms described by Marques (1984), Trouillier et al. (1996), McGeorge et al. (1997), and Yokoyama (1997). The possible identity of spores found by us with the aurantiactinospores described by El-Matbouli et al. (1992), Grossheider and Körting (1992), and Benajiba and Marques (1993) could not be evaluated, as the authors did not give spore dimensions in these works. At the same time, two of the three aurantiactinomyxon types found in Lake Balaton correspond to the types found by us in a previous survey in a fish pond. In this way we have identified type 1 and type 3 from Lake Balaton with type 10 and type 5 from the fish pond, respectively (El-Mansy et al., 1998*b*).

A paper providing general and consistent guidelines on the description of actinospores on their own or as the alternative form of myxosporeans has been published only quite recently (Lom et al., 1997*b*). This is why it is often difficult to compare the forms found by us with those described in earlier works. It is to be hoped that in the future all descriptions will conform to the general guidelines, which would make it easier to compare the results obtained by different researchers. Identification of a species and its systematic position will be easier by the molecular biological methods applied by Andree et al. (1997) and Andree et al. (in press).

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OCCURRENCE OF SKRJABILLANID NEMATODES IN FISHES OF HUNGARY AND IN THE INTERMEDIATE HOST, *ARGULUS FOLIACEUS* L.

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Histozoic and coelozoic skrjabillanid-type nematodes belonging to the genera *Skrjabillanus, Molnaria, Sinoichthyonema, Esocinema, Daniconema* and *Lucionema* are recorded from the subcutaneous tissues, fins, swimbladder and abdominal cavity of different fish species living in natural waters and fish farms of Hungary. In addition to the nine taxonomically identified parasite species, one *Skrjabillanus* sp., two *Molnaria* spp. and three *Esocinema* spp. were identified to the genus level only. The histozoic larval stages of a *Molnaria, Daniconema* and *Lucionema* species each, living in a site different from that of the imagoes, were also detected. The presence of closely not identified first- to third-stage skrjabillanid larvae was demonstrated in 26.3% of the parasitic carp lice (*Argulus foliaceus* L.) collected from the eight fish species.

Key words: Nematoda, Skrjabillanidae, Lucionematidae, Daniconematidae, larval stages, fish hosts, *Argulus foliaceus*, intermediate host

Since the first skrjabillanid nematode, *Skrjabillanus tincae* was described by Shigin and Shigina in 1958, the number of known species belonging to that category has increased substantially. In his monograph, Moravec (1994) reported seven species of four genera (*Esocinema, Molnaria, Sinoichthyonema, Skrjabillanus*) of the Skrjabillanidae family from Europe. *Skrjabillanus cyprini* recently described from the common carp (Molnár and Moravec, 1997) is a further addition to this group. Although *Daniconema anguillae* Moravec et Køie, 1987 assigned to the Daniconematidae family and the most recently detected *Lucionema balatonense* Moravec, Molnár et Székely, 1998 classified into the Lucionematidae family cannot be considered skrjabillanid nematodes from the morphological and taxonomic point of view, they show many similarities to skrjabillanids in their development and site in the host and, therefore, can be described with the adjective 'skrjabillanid' in the broader sense. Relatively few data are available in the literature on the occurrence of these parasites. However, from the results re-

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ported by the few specialists dealing with the subject (Shigin and Shigina, 1958; Molnár, 1966; Mészáros, 1968; Molnár, 1970; Tikhomirova, 1970; Moravec, 1971; Garkavi, 1972; Wierzbicka and Wierzbicki, 1973; Tikhomirova and Rudometova, 1975; Moravec, 1977; Tikhomirova, 1980; Moravec and Køie, 1987; Molnár and Moravec, 1997; Moravec et al., 1998) it seems likely that these parasites are very common and often remain unnoticed only because of the inadequate examination techniques used.

The extrapiscine development of skrjabillanid nematodes was first studied by Rudometova (1974, 1975) and Tikhomirova (1975, 1980). These authors reported that the second- and third-stage larvae of *Skrjabillanus* and *Sinoichthyonema* species developed in the species *Argulus foliaceus* L. and *A. coregoni* Thorell (Branchiura) as intermediate hosts.

Since the first description of *Skrjabillanus scardinii* Molnár, 1966 and *Molnaria intestinalis* (Dogiel et Bychowsky, 1934) (Molnár, 1966), of the dracunculoids of extraintestinal development only the species *Skrjabillanus schigini, Sinoichthyonema amuri* and *Daniconema anguillae* have been detected in Hungary (Molnár, 1989; Molnár and Moravec, 1994; Molnár, 1997), apart from the occurrence of *Anguillicola crassus* and *Philometra* spp. having a different type of development. Recently, however, two new parasite species, *Skrjabillanus cyprini* Molnár et Moravec, 1997 and *Lucionema balatonense* Moravec et al., 1998 have also been recorded (Molnár and Moravec, 1997; Moravec et al., 1998).

The objective of this paper is to demonstrate that skrjabillanids are much more prevalent in Hungarian fishes than is reflected by data of the literature, and this high prevalence manifests itself also in the intensive infection of the intermediate host *Argulus foliaceus*.

Materials and methods

The fish species included in the survey originated from different natural waters of Hungary, first of all from Lake Balaton, from the Kis-Balaton Water Reservoir and from backwaters of the River Körös. In the case of common carp (*Cyprinus carpio*) and grasscarp (*Ctenopharyngodon idella*), numerous fish obtained from fish farms were also dissected. Carp lice (*Argulus foliaceus*) were collected from fish originating from Lake Balaton, Kis-Balaton, and fish farms. After catching them, the fish were separated by species and transported to the laboratory for examination in water supplied with oxygen, in plastic bags. The investigations have been conducted continuously for several years, but the data reported in this paper include only the results obtained between July 1995 and November 1997, i.e. in a period when the technique suitable for the detection of skrjabillanids was consistently applied during the fish dissections.

In the 2.5-year period of study, a total of 656 specimens of 17 fish species were examined for the occurrence of skrjabillanid-type nematodes. The abdominal cavity and the swimbladders were examined for infection in all fish specimens, while the scales and the subcutaneous connective tissue were checked only occasionally.

After killing the fish, the fins, scales and gills were removed and placed into 0.6% physiological saline in a Petri dish. The abdominal cavity was opened, the inner organs were placed into saline, and the cavity was rinsed with the same solution. In the first step, the still moving nematodes washed out from the abdominal cavity were collected in a Petri dish. The parasites located on or under the abdominal serous membranes were released without hurting the gut, by carefully separating the abdominal organs. The serosa was peeled off the swimbladder. After inspecting the viscera, the skin was peeled off the fish, placed into saline in a tumbler, then after isolation for 1–2 h the nematodes released from the subcutaneous connective tissue were collected. To collect the fin-parasitic worms, the folds of the cut-off fins were torn into two halves in their entire length, starting from the base. From the scales, the skin-like layer covering the inner surface was removed. Attempts were made to complete the examinations within 3 h of killing the fish, as the otherwise agile movement of the worms in the tissues and in the solution gradually ceased over time, in which case the hair's-breadth thin, small-sized worms became unrecognisable even in intensive infection. The collected nematodes were either placed directly into lactophenol and fixed as slide preparations, or were conserved in 70% alcohol.

A systematic survey of the occurrence of skrjabillanid larvae in carp lice was carried out only in 1997, when 585 *Argulus* specimens collected from 8 fish species were examined for infection.

The *Argulus* specimens were collected from the fish before the fish were placed into flow-through water. The specimens that had fallen off the fish during transportation were also collected from the water of the plastic bags. The still living, transparent *A. foliaceus* specimens were examined under a coverslip at 100- to 200-fold magnifications of the microscope, and the larvae moving in them were counted and recorded. The larvae present in less transparent crustacean specimens containing eggs or spermatozoa were counted after partial destruction of the crustacean's body by strong pressure exerted on the coverslip. Only a certain proportion of the larvae released from *Argulus* were fixed. A proportion of the nematode larvae found in the carp lice were recorded on videotape, then the video images were transformed into digitalised pictures (Székely, 1997).

The species distribution and the number of fish and crustaceans examined for skrjabillanid infection and the results obtained are presented in Tables 1 and 2.

Table 1

Occurrence of skrjabillanid nematodes in Hungary

Parasite species	Host	Period of examination	Locality	Site of parasite	No. of fish examined	No. of fish infected	Range of intensity (mean int.)
Molnaria intestinalis	*Scardinius erythrophthalmus*	1995–1997	LB, KB, FF	A. c.	30	24	1–8 (3)
Molnaria sp.	*Aspius aspius*	1995–1997	LB, KB	A. c., sb	20	12	1–21 (6)
Molnaria sp.	*Pelecus cultratus*	1996–1997	LB	A. c.	27	8	1–7 (4)
Molnaria sp. larva	*Pelecus cultratus*	1996–1997	LB	Fins	27	18	8–100 (47)
Sinoichthyonema amuri	*Ctenopharyngodon idella*	1995	FF	A. c.	10	10	6–9 (8)
Skrjabillanus scardinii	*Scardinius erythrophthalmus*	1995–1997	LB, KB, FF	Sb, skin	30	20	1–6
Skrjabillanus cyprini	*Cyprinus carpio*	1996–1997	LB, KB, FF	Scales	14	7	1–8 (3)
Skrjabillanus schigini	*Ctenopharyngodon idella*	1995	FF	Sb	10	10	1–7 (4)
Skrjabillanus tincae	*Tinca tinca*	1997	KB	Sb	2	2	1–3
Skrjabillanus sp.	*Abramis brama*	1996	LB	Scale	28	1	1
Lucionema balatonense[*]	*Stizostedion lucioperca*	1995–1997	LB	Sb	160	3	5–30 (23)
Lucionema balatonense larva[*]	*Stizostedion lucioperca,*	1995–1997	LB	Sb	160	5	1–32 (25)
	S. volgense	1996	LB	Sb	24	2	1–1
Daniconema anguillae[**]	*Anguilla anguilla*	1995–1997	LB	Fins, sb	82	6	1–2
Daniconema anguillae larva[**]	*Anguilla anguilla*	1995–1997	LB	Fins, sb	82	45	10–500
Esocinema bohemica	*Esox lucius*	1997	KB	Sb	7	2	1–2
Esocinema sp.	*Aspius aspius*	1996–1997	LB, KB	Sb, skin	20	13	1–9 (4)
Esocinema sp.	*Pelecus cultratus*	1996–1997	LB	Sb, skin	27	13	1–7 (3)
Esocinema sp.	*Abramis brama*	1997	LB	Skin	4	1	1

LB = Lake Balaton; KB = Kis-Balaton Water reservoir; FF = Fish Farm (Biatorbágy); A. c. = abdominal cavity; sb = swimbladder; [*]Classified into the Lucionematidae family; [**]Classified into the Daniconematidae family

Table 2

Infection of *Argulus foliaceus* specimens collected from different fish species with the larvae of skrjabillanid nematodes

Fish species	Collection		No. of carp lice		Prevalence of infection, %	Intensity of infection, ranges (mean)
	Place	Date	examined	infected		
Cyprinus carpio	Kis-Balaton	23.08.96	6	6	100	1–8 (4)
	Kis-Balaton	03.09	6	6	100	1–3 (2)
	Kis-Balaton	04.09	10	7	70	1–4 (2)
	Kis-Balaton	07.08.97	18	7	39	1–30 (7)
	Kis-Balaton	10.10	76	18	24	1–5 (3)
	Hortobágy	16.10	67	16	24	1–7 (4)
	Biatorbágy	21.10	23	4	17	1–3 (2)
	Biatorbágy	29.10	62	10	16	1–8 (3)
Aspius aspius	Balaton	26.09.96	1	1	–	5
	Kis-Balaton	07.08.97	22	4	18	1–2 (1.7)
	Kis-Balaton	10.10	12	4	17	1–7 (2.7)
	Biatorbágy	29.10	12	2	–	2–9
Aristichthys nobilis	Biatorbágy	29.10	9	2	22	2–2
Abramis brama	Balaton	02.10	1	0	–	
	Kis-Balaton	10.10	6	1	–	2
Ctenopharyngodon idella	Kis-Balaton	10.10	2	2	–	2–4
	Biatorbágy	29.10	14	5	36	1–3 (2)
Tinca tinca	Kis-Balaton	23.08.96	3	2	–	1–2
Stizostedion lucioperca	Kis-Balaton	21.07	8	3	–	1–2
	Kis-Balaton	03.09	3	1	–	3
	Kis-Balaton	26.09	1	1	–	2
	Kis-Balaton	07.08.97	30	4	13	1–2
	Balaton	02.10	3	0	–	
	Kis-Balaton	10.10	66	13	20	1–4 (1.7)
	Kis-Balaton	20.10	4	2	–	3–5
	Biatorbágy	21.10	12	4	33	1–2
	Biatorbágy	29.10	36	8	22	1–13 (2.6)
Esox lucius	Biatorbágy	21.10	3	1	–	1
	Biatorbágy	29.10	50	14	28	1–18 (4.6)
Mixed species	Kis-Balaton	07.08	19	6	32	1–10 (4.5)
Total	–	–	585	154	26.3	–

Results

I. Surveys on fish

Skrjabillanid-type nematodes were detected from 11 fish species (Table 1). Of the fish species included in the survey with at least 10 specimens examined, 252 specimens of 10 species proved to be free of skrjabillanid infection: *Alburnus alburnus* (30), *Barbus barbus* (17), *Blicca bjoerkna* (30), *Carassius auratus gibelio* (62), *Rutilus rutilus* (11), *Aristichthys nobilis* (14), *Hypophthalmichthys molitrix* (14), *Silurus glanis* (16), *Perca fluviatilis* (16), *Gymnocephalus cernuus* (42).

As regards the skrjabillanid species detected, the results presented in the table must be complemented with the following data:

(1) *Skrjabillanus cyprini* Molnár et Moravec, 1997. This parasite occurred in common carp (*Cyprinus carpio*) in natural waters and fish farms alike. Its typical site is the subsquamal space between the cartilaginous plate of the scales and the thin epithelial layer covering them on the inside. Less commonly the parasite was found also under the scales of the mirror carp, a species having only a few scales.

(2) Numerous breams (*Abramis brama*) were examined, but only a single *Skrjabillanus* female was found on one occasion. That specimen was found subsquamally, in the same location as *S. cyprini*, and resembled the latter also in shape and size; however, its fixation was unsuccessful. In the abdominal cavity and swimbladder of the breams examined there was 100% prevalence of infection with another dracunculoid nematode, *Philometra ovata* (Zeder, 1803). Mostly specimens of arrested development were found, and mature nematodes were only rarely detected.

(3) In several years old specimens of the rudd (*Scardinius erythrophthalmus*), *Molnaria intestinalis* (Dogiel et Bychowsky, 1934) and *Skrjabillanus scardinii* Molnár, 1966 occurred almost always as concurrent infection. At the same time, in 2–3 years old fish usually the specimens of only one of these parasite species could be detected.

(4) In the grasscarp (*Ctenopharyngodon idella*), the species most closely related to the rudd, the occurrence of also two nematode species, *Skrjabillanus schigini* Tikhomirova et Rudometova, 1975 and *Sinoichthyonema amuri* (Garkavi, 1972) was commonly recorded.

(5) The tench (*Tinca tinca*) was found to be parasitised by *S. tincae* Schigin et Schigina, 1958, the type species of the *Skrjabillanus* genus. It has been detected in Hungary for the first time in this survey.

(6) In asp (*Aspius aspius*) specimens exceeding 30 cm in size, the occurrence of three dracunculoids was commonly recorded. Specimens of *Philometra*

kotlani (Molnár, 1969) and a hitherto not closely identified *Molnaria* sp. were regularly found in the abdominal cavity. In the swimbladder serosa and subcutaneous connective tissue of the same fish species specimens of an *Esocinema* sp. occurred. Occasionally all three parasite species were simultaneously present; however, in the majority of cases specimens of the *Molnaria* sp. could not be detected in asps infected by *Philometra kotlani*.

(7) In the chekhon (*Pelecus cultratus*) a *Molnaria* and an *Esocinema* species could be detected in a similar site as in the asp.

(8) In the pikeperch (*Stizostedion lucioperca*), the specimens of *Lucionema balatonense* could be detected in only three cases. These nematodes, which are similar to skrjabillanids, have been described and assigned to the independent Lucionematidae family belonging to the Dracunculoidea superfamily by Moravec, Molnár and Székely (1998). In the three infected pikeperch specimens, however, more than 30 nematodes (all females with only a single male present) were found freely in the swimbladder.

(9) *Esocinema* spp. were detected in four fish species (*Esox lucius, Pelecus cultratus, Aspius aspius, Abramis brama*). Of them, the nematodes found in the pike were identified with the species *Esocinema bohemicum* Moravec, 1977, while those found in cyprinids were determined to the genus level only. In asp and chekhon the worms were detected also in the swimbladder, while in pike and bream they were isolated only from the subcutaneous connective tissue. Such nematodes were not found, however, under the skin of *Blicca bjoerkna, Rutilus rutilus, Leuciscus idus, Cyprinus carpio, Carassius carassius* and *Silurus glanis* specimens examined by a similar technique.

(10) *Daniconema anguillae* Moravec et Køie, 1987 was detected in eels in both abdominal and fin site. Infection of the fins was found to be more common.

II. Infection of fish with skrjabillanid larvae

Infection with skrjabillanid larvae occurred in four fish species, i.e. in the eel, pikeperch, Volga pikeperch, and chekhon.

(1) Infection of the fins of eel with third-stage *Daniconema anguillae* larvae has been reported in detail by Molnár and Moravec (1994). That infection proved to be common also in the present survey, affecting about 40% of the eels examined.

(2) The 360–400 μm long first-stage larvae of *Lucionema balatonense* were present under the serosa of the swimbladder in 5 out of the 160 pikeperch and in 2 out of the 24 Volga pikeperch specimens examined.

(3) Coiled-up nematode larvae were detected in the caudal, anal and dorsal fins of 18 out of the 27 chekhons examined. They were located in the skin fold between the fin rays and less often in the fin rays themselves (Figs 1 and 2).

By their characteristic buccal capsule they could be identified with the third-stage larvae of stagnating development of the *Molnaria* sp. parasitic in the abdominal cavity of chekhon. The prevalence of infection was 66% and its intensity varied between 1 and 450 (mean: 47).

III. Survey of Argulus foliaceus *for infection with nematode larvae (Table 2)*

In Hungary, the infection of *A. foliaceus* with nematode larvae was first observed in 1995, when infection of the swimbladder of pikeperch with *Lucionema balatonense* larvae was first detected. At that time, a young larva with tapered caudal end (Fig. 3) was detected in an *Argulus* specimen found on an infected fish. In that year, 7 out of the 9 *Argulus* specimens collected from pikeperch were found to be infected with larvae. In the subsequent year (1996), 33 out of the 44 *A. foliaceus* specimens collected from pikeperch, common carp, asp and tench at random proved to be infected with skrjabillanid-type larvae.

In the present survey conducted in 1997, infection with nematode larvae was detected in 26.3% of the *Argulus* specimens examined. The highest prevalence of infection was found in carp louse specimens collected from grasscarp. In the majority of cases the intensity of infection was restricted to one or two larvae; however, there were *Argulus* specimens infected with as many as 18 and 30 larvae, respectively.

The larvae occurred freely in the body cavity of the carp louse and were constantly changing their location. Young larvae preferred staying in the suckers but commonly occurred also in the crustacean's legs (Fig. 3), uropods (Fig. 4), and other sites of the body (Fig. 5). In their size and tapered caudal ends (Fig. 3) the youngest larvae resembled the first-stage larvae developing in the body of imago *Skrjabillanus* and *Molnaria* females; however, after the first moulting they lost their tapered tail (Fig. 6 and inset of Fig. 6) and both their head and tail ends became rounded. Larger specimens were detected around the stylet or in its cavity (Figs 7 and 8). By their tapering, slightly bent tail and the primordium of the buccal capsule, some of these advanced larvae resembled the larvae isolated from the fins of chekhon (Fig. 2) and displayed a typical *Skrjabillanus* character.

Discussion

The results of the survey indicate that skrjabillanid nematodes assigned to the order Dracunculoidea are extremely common parasites of Hungarian fishes and can consistently be detected in some fish species if a suitable examination technique is used.

Skrjabillanus cyprini seems to be specific for the common carp, as it could not be found in the closely related gibel carp (*Carassius auratus gibelio*). The detection of a closely not identified specimen from the same site in the bream, however, queries that explicit statement.

According to data obtained over a period of several years, *Skrjabillanus scardinii* and *Molnaria intestinalis* infection can be considered almost universal in the rudd (Molnár, 1989). It is striking, therefore, that no skrjabillanids could be found in *Rutilus rutilus*, another cyprinid belonging to the Leuciscinae subfamily, although the species *Molnaria leucisci* had been described from another member of that subfamily, *Leuciscus idus* (Agapova, 1963).

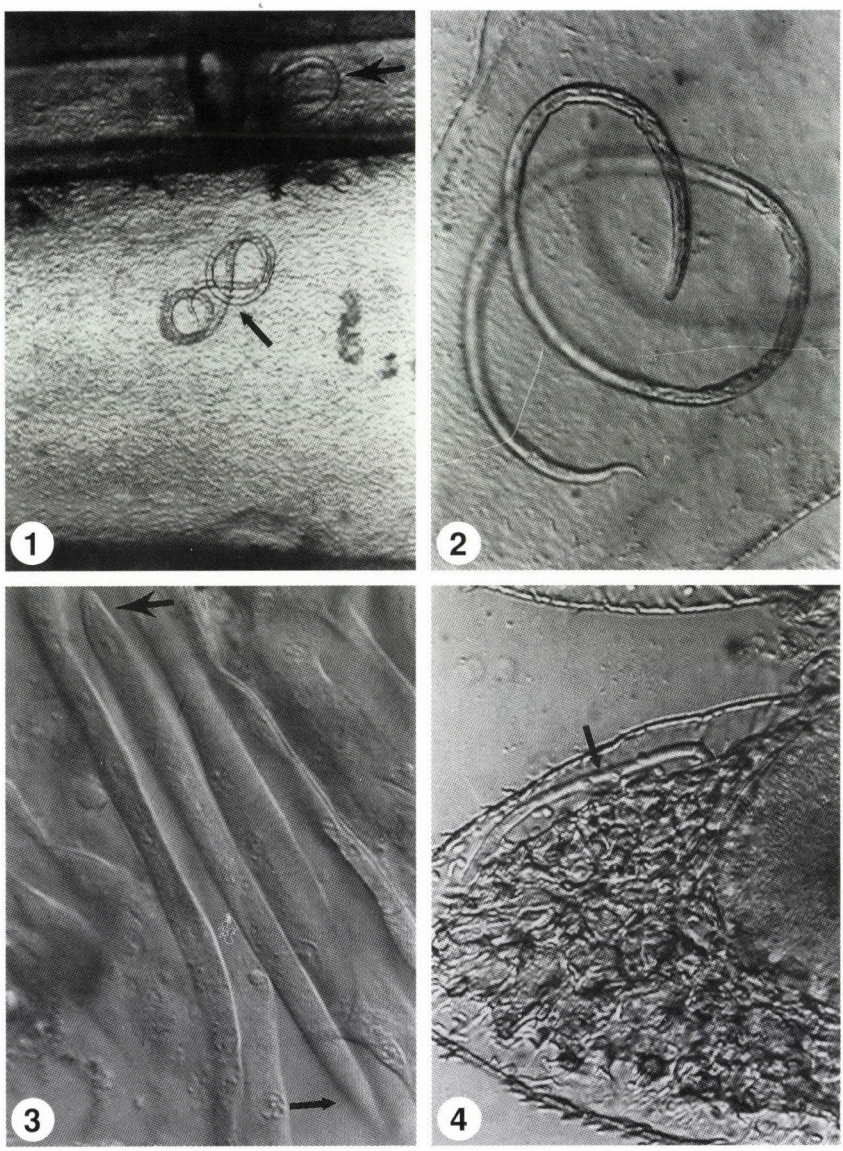
Mészáros (1968) had already detected the *Molnaria* species parasitising the asp in Lake Balaton, and identified it with *M. intestinalis*. Other authors (Tikhomirova, 1980; Boni et al., 1990; Moravec and Scholz, 1991) also claimed to have found *M. intestinalis* in several cyprinids. Despite this fact, the specimens found are regarded only as *Molnaria* sp. until more detailed studies are carried out.

The same applies to the *Molnaria* sp. found in the chekhon. The imagoes greatly resemble the species *M. intestinalis* in their morphological characters; however, the unique fin location of the third-stage larvae, found neither for *M. intestinalis* of the rudd nor for the *Molnaria* sp. of the asp, makes the diagnosis difficult. The question arises whether the specific location of the larvae can be explained by the biological properties of a species different from *M. intestinalis* or by the especially high level of infection found in the chekhon.

As regards the *Esocinema* species found in cyprinids it has to be elucidated whether the species found are identical with the species *Esocinema bohemicum* described from the type host, the pike. If yes, this would mean a loose host specificity which is less typical of skrjabillanids.

Two species belonging to the Anguillicolidae and Daniconematidae families of the Dracunculoidea superfamily are common parasites of the eel (*Anguilla anguilla*). Of them, only *Daniconema anguillae* is characterised by a location similar to that of *Skrjabillanus* species and by the intermittent excretion of larvae from the viviparous females. The unique location of the parasite's larvae was described by Molnár and Moravec (1994). Subsequently, Molnár (1997) reported the previously unknown fin location of the imagoes. The relatively frequent occurrence of the larvae indicates that this parasite is rather common in the eel despite the fact that the imagoes are rarely detectable. The swimbladder and the fins are likely to represent only one of the possible site, and worms colonising the intermuscular connective tissue or other sites of the organism remain hidden from the examiner.

The survey clearly proves that skrjabillanids develop via the carp louse as intermediate host, as has been demonstrated by Tikhomirova (1970, 1975) and Rudometova (1974, 1975) for *Skrjabillanus scardinii, Molnaria intestinalis* and



Figs 1–2. Video-recorded images of the fin of chekhon infected with *Molnaria* larvae
Fig. 1. Molnaria larva in the fin ray (a) and skin fold between the fin rays (b) of the chekhon. × 60
Fig. 2. Third-stage *Molnaria* larva released from the fin ray of a chekhon. × 200
Figs 3–8. Video-recorded images of carp lice infected with skrjabillanid larvae;
Fig. 3. Young skrjabillanid (probably *Lucionema*) larva from the leg of a carp louse (*Argulus foliaceus*) collected from pikeperch. Note the blunt cephalic end (large arrow) and tapered caudal end (small arrow) of the larva. × 800
Fig. 4. Skrjabillanid larva (arrow) in the uropod of an *Argulus foliaceus* collected from common carp. × 60

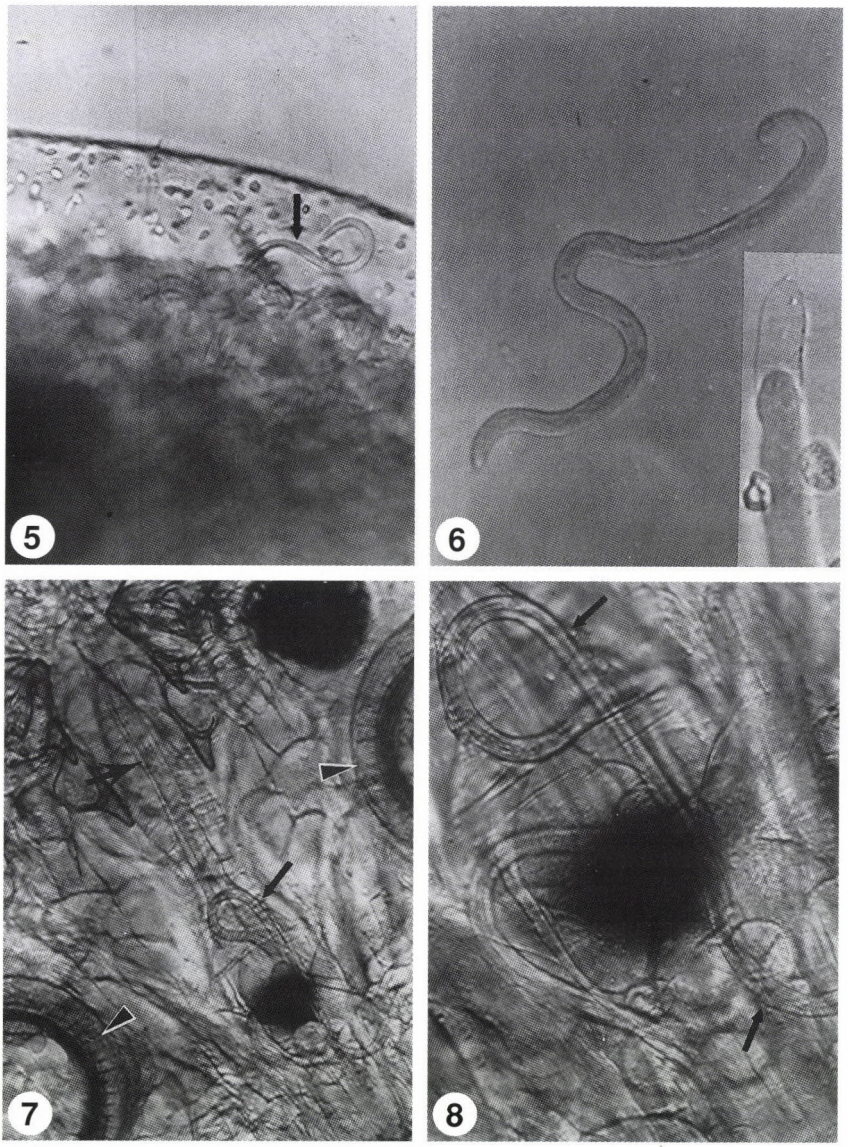


Fig. 5. Skrjabillanid larva (arrow) in the lateral part of the body of a carp louse collected from common carp. × 40

Fig. 6. Young skrjabillanid larva released from a carp louse. × 150. Inset: Larva released from a carp louse bearing a moulted cuticle at its cephalic end. × 300

Fig. 7. Skrjabillanid larva of advanced development (small arrow) in the stylet of a carp louse. The suckers of the carp louse are marked with an arrowhead. × 40

Fig. 8. Skrjabillanid larva of advanced development, wriggling in the basal part of the stylet of a carp louse (arrows). × 120

Sinoichthyonema amuri. At the same time, the present investigations do not allow us to draw further conclusions, as the carp louse is well known to change hosts several times during its life. Because of that change of hosts it is impossible to prove that the larva found in the crustacean is identical with the larval form of the skrjabillanid sp. or spp. occurring in the fish host, although infection of the carp louse by young stages is suggestive of a very likely infection of the host. Larvae of extremely variable shape and size were obtained from the carp lice. Obviously only a proportion of these corresponded to the larval stages of species belonging to the genera *Skrjabillanus, Molnaria* and *Sinoichthyonema*, which have been proved to develop in *Argulus* intermediate host. These larval stages assumedly included those of *Esocinema* and *Daniconema* species. At the same time, the present survey has demonstrated that the majority of carp lice collected from fish harbours a natural infection and, therefore, are unsuitable for use in reliable experiments. This problem could only be solved by the laboratory culture of *Argulus* intermediate hosts reared on parasite-free fish that have not been infected earlier.

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PHILOMETRA KARUNENSIS SP. N. (NEMATODA: PHILOMETRIDAE) FROM *BARBUS SHARPEYI* (PISCES) IN FRESHWATERS OF SOUTHWEST IRAN

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During a study on some *Barbus* species (Pisces: Cyprinidae) of the Mesopotamian fauna region (the south-western part of Iran) a new nematode species, *Philometra karunensis* sp. n., was found in the swimbladder (under the serosa cover) and abdominal cavity of *Barbus sharpeyi* (Günther). Males distinctly differ from the known species in the length of the spicules and gubernaculum, while subgravid females are very similar to those of *Philometra* spp. known from the abdominal cavity of cyprinid fishes.

Key words: Nematoda, *Philometra*, *Barbus sharpeyi*, Iran

During a survey on the nematode fauna of Iranian fishes, large numbers of worm species were collected and identified. Among the nematodes recovered, a new *Philometra* species was studied in detail.

Papers on the occurrence of philometrid nematodes in Iran were first published by Ebrahimzadeh and Nabawi (1975) and Moghainemi and Abasi (1992). These authors identified the *Philometra* specimens found in freshwater fishes only to the genus level, but gave no data on their morphology and seasonality. Molnár and Pazooki (1995), who studied *Philometra* infection in Iranian barbels (*Barbus* spp.) from the Mesopotamian region, found these parasites in *Barbus sharpeyi* (Günther, 1874) and *B. luteus* (Heckel, 1843). They could not specifically identify the specimens but supposed that these worms represented a new species. However, having obtained the male and subgravid female specimens from different species of fish, they abstained from formally establishing one.

More data are available on the occurrence of philometrid nematodes from the Iraqi side of the Tigris River system. Herzog (1969) was the first to find female specimens of a *Philometra* sp. in the abdominal cavity of *Barbus luteus*, then Ali et al. (1987) reported the occurrence of a *Philometra* sp. in the abdomi-

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nal cavity of *Barbus grypus* (Heckel, 1843), which they identified as *P. abdominalis* Nybelin, 1928.

Philometrid nematodes are common parasites of freshwater and marine fishes. Their systematics is mainly based on the morphological characteristics of female worms, because only gravid females have been described for most of the known species. On this basis, Moravec and Shaharom-Harrison (1989) distinguished 11 genera in the family Philometridae, of which species of the genus *Philometra* are the most numerous and widespread.

The purpose of this paper, partly based on data by Molnár and Pazooki (1995), is to describe a new *Philometra* species from *Barbus sharpeyi.*

Materials and methods

Philometra specimens were collected during parasitological surveys involving fish species of the Karun River, Shadegan Marsh and Hoor-Elazim Marsh (Tigris River region, Persian Gulf system) between 1993 and 1996. A total of 62 *Barbus sharpeyi* (Günther, 1874), 2–4 years old, were dissected for parasites. A general parasitological dissection was performed. In order to detect *Philometra* specimens, the abdominal cavity and the swimbladder were checked with meticulous care. Organs of the abdominal cavity were examined in 0.65% saline and the cavity was rinsed with this solution. The occurrence of *Philometra* stages in this washing fluid was checked in a Petri dish. Males and retarded females were looked for in the serous membrane of the second sac of the swimbladder, which was peeled off the sac wall.

Nematodes found were placed into 4% formalin in physiological saline and preserved until their detailed study. The isolated *Philometra* specimens were cleared in lactophenol to examine their surface and inner structure. The structure of the cephalic papillae and the buccal region was studied in *en face* view after decapitating the subgravid females with a sharp blade (Moravec, 1994). Measurements and drawings were taken using an Olympus microscope.

Results

During the survey conducted between 1993 and 1996, 13 out of 62 *B. sharpeyi* specimens (21%) proved to be infected by different *Philometra* stages. Three of the infected *B. sharpeyi* harboured 5 subgravid females in the abdominal cavity and 10 of them were infected by 9 males and 13 mature non-gravid females under the serosa of the swimbladder. The males and the non-gravid females were found in *B. sharpeyi* from July to mid-September. Subgravid females occurred from the

end of November till March. By its characteristic morphological features this species proved to be new for science and is described as follows.

***Philometra karunensis* sp. n. (Fig. 1)**

Type host: *Barbus sharpeyi* (Günther, 1874) (Cyprinidae, Cypriniformes)

Type locality: Karun River, Shadegan and Hoor-Elazim Marsh in Southwest Iran.

Site of infection: Females in the abdominal cavity, males under the serosa of the swimbladder.

Type material: Holotype and allotype deposited in the helminthological collection of the Zoological Department, Hungarian Natural History Museum, Budapest. Paratypes are in the collection of the senior author.

Etymology: The name of the species was given after the Karun River

Males (7 specimens; measurements of holotype in parentheses): Body thread-like, whitish, with smooth cuticle. Head end rounded. Papillae and amphids not seen by light microscope. Body length 2.6 to 3.2 (2.64) mm, maximum width 0.072–0.090 (0.084) mm. Oesophagus narrow, 0.540–0.570 (0.570) mm long, cylindrical, muscular, with a bulb-shaped inflation at anterior end. Posterior portion of oesophagus with oesophageal gland 0.316–0.340 (0.340) mm in length. Posterior end of the body distinctly lobed, showing two papilla-like protrusions laterally. Spicules sclerotized, slender, equal in size, measuring 0.068–0.077 (0.075) mm in length. Their width 0.004–0.005 (0.005) mm at base and 0.002–0.003 (0.003) mm at middle. Gubernaculum 0.043–0.046 (0.045) mm long, bent sclerotized plate with lancet-shaped posterior end (Figs 1D–F).

Subgravid females (4 specimens; measurements of allotype in parentheses): Body of live female red in colour, cylindrical, 65–105 (82) mm long and 0.72–1.5 (1.2) mm wide tapering slightly to both ends; cuticle smooth; body ends rounded. Oral opening circular, with three flat oesophageal lobes, surrounded by small lateral amphids and cephalic papillae arranged in two circles. Four slightly elevated lobes, each bearing 2 submedian papillae, arranged in outer circle and 4 small submedian papillae located in inner circle. Oesophagus relatively narrow, somewhat expanded at anterior end and provided with dorsal oesophageal gland with a giant cell nucleus at its mid-length. Length of oesophagus 1.42–1.85 (1.75) mm. Nerve ring encircling oesophagus at 0.24–0.33 (0.32) mm distance from anterior extremity. Intestine straight, displaced laterally; its posterior end forming ligament attached ventrally to body wall. Posterior end of body rounded with two small lateral papilla-like projections. Vagina and vulva absent. Uterus occupying almost the whole space of body, being filled with eggs 0.025–0.029 mm in diameter (Figs 1A–C).

Mature females (4 specimens): White in colour, cuticle smooth. Length of body 2.8–13 mm, width 0.10–0.54 mm. Body nearly uniform in diameter throughout, narrowing slightly toward both ends. Posterior end terminates in two distinct

lobe-like projections. Mouth very small, circular, papillae or amphids not seen by light microscope. Oesophagus narrow, inflated at anterior end. Ratio of oesophagus to body length 1 to 11. Ratio of oesophageal gland length to total length of oesophagus 76%. Vulvar opening lies in posterior third of body. It is circular in shape, continuing anteriorly in a 0.063 to 0.071 mm long sclerotized vagina 0.01 mm in diameter. Uterus uniformly thick sac, filled up with eggs 0.009 mm in diameter; it continues in tubular ovaries in both directions. Anterior ovary starts from uterus near centre of oesophagus, returns and ends close to mouth of the intestine. The posterior ovary forms loops at rectum and, returning, ends bluntly near uterus (Figs 1G–I).

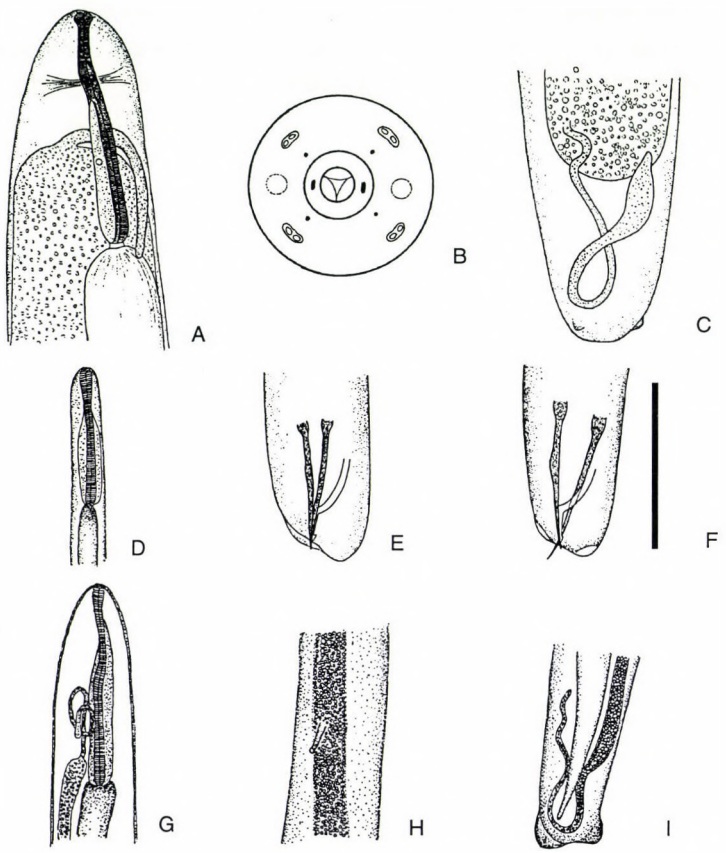

Fig. 1. Schematic drawing of *Philometra karunensis*. **A** – cephalic end of a subgravid female, lateral view. Bar = 1.2 mm; **B** – cephalic end of a subgravid female, apical view. Bar = 1 mm; **C** – caudal end of a subgravid female. Bar = 1.2 mm; **D** – cephalic end of a male. Bar = 0.4 mm; **E** – caudal end of a male (holotype) with spicules and gubernaculum. Bar = 0.1 mm; **F** – caudal end of a male (paratype) with spicules and gubernaculum. Bar = 0.1 mm; **G** – cephalic end of a mature (retarded) female. Bar = 0.5 mm; **H** – region of vulva. Bar = 0.5 mm; **I** – caudal end of a matured female. Bar = 0.5 mm

Discussion

During the present investigation, nematodes of different developmental stages characteristic of the genus *Philometra* were found in *Barbus sharpeyi.* Subgravid females of this *Philometra* resemble females of *P. abdominalis* Nybelin, *P. ovata* Zeder and *P. kotlani* Molnár, the three species common in the abdominal cavity of cyprinids, and they practically do not differ from the latter. However, the differences in their host preference seem to be more important. *P. kotlani* is a specific parasite of *Aspius aspius*. *P. ovata* occurs mainly in *Abramis* spp. and *Rutilus* spp.. *P. abdominalis* can complete its developmental cycle only in *Phoxinus phoxinus* and in *Gobio* spp. In other fish species including *Barbus meridionalis petenyi* only retarded forms of *P. abdominalis* were found in Hungary (Molnár, 1967, 1969). No *Philometra* spp. have been reported from *Barbus barbus*, a well-studied fish of European rivers (Vismanis et al., 1987; Moravec, 1994). While the females of philometrids from cyprinids show only slight morphological differences, the males of the known species differ significantly in the length of the spicules and gubernaculum. The spicules of specimens found in *Barbus sharpeyi* are equal and significantly shorter than those of *P. ovata, P. abdominalis* and *P. kotlani,* but resemble *P. rischta*, a non-abdominal parasite of the bleak and *P. cylindracea* and *P. kobuleji*, the parasites of the North American yellow perch and white sucker, respectively (Molnár and Fernando, 1975*a*, 1975*b*).

Data presented by Iraqi and Iranian authors (Herzog, 1969; Ebrahimzadeh and Nabawi, 1975; Ali et al., 1987; Moghainemi and Abasi, 1992; Molnár and Pazooki, 1995) indicate that *Philometra* spp. are common inhabitants in different fishes of the genus *Barbus* in the rivers of the Mesopotamian region. It is remarkable that in other parts of the world with a better-known parasite fauna relatively few philometrid nematodes have been recorded from *Barbus* spp. As concerns European *Barbus* spp. there have been only two reports on the occurrence of a *Philometra* species. Molnár (1967, 1969) reported retarded females and males of *Philometra abdominalis* in the swimbladder serosa of *Barbus meridionalis petenyi* in Hungary.

Before the present investigations Herzog (1969) and Ali et al. (1987) mentioned the occurrence of *Philometra* species in barbels in Iraq. Ali et al. (1987) recorded female specimens. Identification of the specimens found by them as *P. abdominalis* seems to be a mistake, as no gravid female of this species has been detected in European barbels. Although *Philometra* spp. have less strict host specificity in cyprinids, they usually show a distinct host selection.

In Europe *Philometra abdominalis* Nybelin, 1928 is common in fishes of the genera *Gobio* and *Phoxinus*, while *P. ovata* Zeder, 1803 infects *Abramis* and *Rutilus* spp. On the other hand, *P. kotlani* Molnár, 1969, *Philometroides cyprini*

Ishii, 1931 and *P. sanguinea* Rudophi, 1819 infect only members of the genera *Aspius, Cyprinus* and *Carassius*, respectively.

The development of nematodes belonging to the Philometridae family markedly differs from that of other nematodes. Significant diversity can be observed in the size of males and females. In contrast to gravid females which are several centimetres long and filled with larvae, the males measure only a few millimetres in length (Molnár, 1967, 1969; Moravec, 1994). As during the one-year period of synchronous development the size of females, but not that of the males, continuously increases, in species description a distinction should be made among mature, subgravid and retarded females. As interpreted by Moravec (1994), the category of mature worms includes females having the same size as males, and possessing a well-developed vulva and vagina. If these remain unfertilised and get stuck in the swimbladder, they can be described by the adjective 'retarded'. Females representing different stages of development, filled with eggs but having an atrophied vagina and vulva can be described as 'subgravid', whereas fully developed females filled with larvae as 'gravid'. In addition to male worms, mature (presumably retarded) females and subgravid females representing different developmental stages were studied during this survey; however, no gravid specimens were collected.

Identification of *Philometra* species is often based on the morphology of gravid females. However, females of some species are practically indistinguishable and, therefore, differences in the morphological characters of males are more significant. The extraordinarily small males, however, are known only from a minor fraction of the species described.

Prior to this study, Molnár and Pazooki (1995) also found *Philometra* infection in Iranian barbels, and their collection comprised both males and females. As, however, subgravid females and males originated from two different fish species (*Barbus sharpeyi, B. luteus*), the authors did not describe a new species.

The collected material indicates that the specimens found in *Barbus sharpeyi* are not identical with the known *Philometra* species and we regard them as a new species. On the other hand, the present material does not allow us to speculate whether or not *Philometra* specimens found in *B. grypus* and *B. luteus* from the same habitat by Iraqi and Iranian authors could be classified as *P. karunensis*, too. In our opinion, this question could be answered either by collecting males and gravid females from the barbels in question or by experimental infection of these fish species.

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3,3',5-TRIIODOTHYRONINE (T_3) UPTAKE AND EXPRESSION OF THYROID HORMONE RECEPTORS DURING THE ADAPTATION TO HYPOTHYROIDISM OF THE BRAIN OF CHICKEN

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Thyroid hormone action in the brain is strictly regulated, since these hormones play a crucial role in the development and physiological functioning of the central nervous system. Hormone kinetics and molecular events at the nuclear receptor level during the adaptation of the brain of chicken to hypothyroidism were simultaneously investigated. Data obtained by Oldendorff's 'single-pass' technique showed a significantly higher labelled 3,3',5-triiodothyronine (^{125}I-T_3) uptake into the brain of surgically thyroidectomized (TX) 2-week-old broilers after 1 week of the surgery in comparison to sham-operated (SH) and T_3 supplemented (TX + T_3) controls in the 10th second after the bolus injection. Telencephalons showed the highest, while cerebellum the lowest uptake intensity in all groups. In a similar arrangement of experiments the expression of the TRα- and TRβ nuclear thyroid receptors in the telencephalon of TX and control chickens was investigated by a semiquantitative RT-PCR study in the following way: cDNAs were standardised using a quantitative RT-PCR-based approach for β-actin, then amplified for thyroid receptors. The level of both the TRα and TRβ coding mRNA was elevated in hypothyroidism. In conclusion, the presented hormone kinetics and TR expression data provide further details of the cellular and molecular events occurring during the adaptation to hypothyroidism of the brain of chicken.

Key words: Brain, chicken, thyroid hormones, thyroid receptors, kinetics of thyroid hormone, uptake

The importance of thyroid hormones in the development and maturation of the central nervous system was stressed already by classical studies (Eayrs, 1960). It also became clear from a line of publications that the brain and certain other tissues were able to avoid intracellular hypothyroidism by several mechanisms in different species (rat: Silva and Mathews, 1984; chicken: Rudas, 1986;

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Rudas, 1989). Three major sites of possible adaptation in the brain entail the altered uptake, cellular handling and distribution, and the elimination of thyroid hormones during hypothyroid states. All three pathways have been proven to exist in chickens, by classical hormone and enzyme kinetics analyses performed by our group (Rudas and Bartha, 1993; Rudas et al., 1993; Rudas et al., 1994).

The present paper attempts to get more information about the adaptation of the chicken brain to hypothyroidism by combining two approaches: (1) hormone kinetics studies obtained by Oldendorff's 'single-pass' technique (see Materials and methods); (2) investigation of nuclear receptor related intracellular handling of thyroid hormones at the molecular level.

Thyroid hormone receptors (TR), belonging to the thyroid/steroid receptor superfamily, were identified as the cellular homologues of the v-erbA oncogene of the avian erythroblastosis retrovirus. Two separate genes were cloned. TRα was first isolated from a chick liver cDNA library (Sap et al., 1986), and later cloned from rat brain as well (Thompson et al., 1987). TRβ was identified in a human placental cDNA library (Weinberger et al., 1986), and later it was cloned also from the chicken kidney (Forrest et al., 1990).

These two receptors bind 3,3',5-triiodothyronine (T_3) with similar affinity, but possess certain amino acid differences in the DNA binding domain both in chicken (Forrest et al., 1990) and rat (Murray et al., 1988). Different subtypes, generated by alternative splicing, were also identified. TRα subtypes (α_2, α_3) cannot bind T_3, as was proved in rats and humans (Izumo and Mahdavi, 1988; Lazar et al., 1988 and 1989). TRβ_2 is a functioning receptor, but its expression is restricted to the pituitary, as was shown in the rat (Hodin et al., 1989). In contrast, in the chicken no C-terminal variants of the thyroid receptor genes could be detected by RNase protection assay (Forrest et al., 1990), but a N-terminal variant, called chicken TRβ_2, was identified during retinal development (Sjoberg et al., 1992). The cloning of thyroid hormone receptors initiated a line of investigations to clarify the molecular events, underlying the adaptation of the brain to the altered thyroid status, which is yet incompletely understood.

The purpose of the present study was to investigate simultaneously the adaptation of the brain of chicken to hypothyroidism by hormone kinetics and receptor expression studies.

Materials and methods

In our first set of experiments (*experiments A through C*) Oldendorff's 'single-pass' technique (Pardridge, 1981) was used to measure the instant uptake of 3,3',5-triiodothyronine by the brain of control and of hypothyroid chickens. In the second set of experiments the adaptation of the chicken brain to hypothy-

roidism was looked at by investigating the nuclear T_3 receptor expression (*experiment D*).

Experiments were done on broiler chickens obtained from a commercial broiler producer (Rákos State Farm, KTKI, Gödöllő, Hungary). The room temperature, water and food supply were adjusted (as in exp. D) according to the instructions of the supplier. Cockerels were used throughout. T_3 and T_4 levels of the sera were monitored in all experiments by radioimmunoassay, according to our routine protocol (Pethes et al., 1978*a*, *b*), to exclude animals with atypical thyroid status in all groups mentioned below.

Since in *experiment A* the Materials and methods were the same as described in our earlier paper (Rudas and Bartha, 1993) only a brief summary is given here.

Animals of experiments A and B were allotted into three groups of 10 individuals each: surgically thyroidectomized (Veresegyházy et al., 1986), TX; sham-operated controls, SH; and thyroidectomized birds with daily T_3 supplementation (as described earlier, Kühn et al., 1984), TX + T_3. Seven days after surgical intervention (on the 14th day after hatching) the birds were anaesthetised with Nembutal (25 mg/kg body weight), immobilised, and the area of the a. carotis interna was exposed on the left side of the neck. One hundred µl of physiological saline with purified labelled hormone (^{125}I-T_3) with traces of bovine serum albumin (0.02 g/L) were injected into the left a. carotis interna within 1 sec as a bolus. In experiment A the birds were decapitated exactly 10 sec after the bolus injection. Parts of the brain (telencephalon, TEL left and right, brain stem + mesencephalon, B + M, and cerebellum, CM) were dissected out as rapidly as possible and were deep-frozen on dry-ice-acetone. Brain samples were analysed for ^{125}I-T_3 in a chromatographic system substantiated earlier (Rudas and Pethes, 1989). The instant iodothyronine uptake was calculated as % of dose of radioactivity/g wet tissue/100 g body weight. We have shown earlier that the average plasma volumes and the protein content of the brain of TX and SH groups do not differ significantly (Rudas et al., 1994).

In experiment B and C the 'single-pass' character of our method was checked in two ways.

Experiment B: ^{131}I-bovine serum albumin (^{131}I-BSA, Izinta Ltd., Budapest, Hungary) was purified by Sephadex 10 gel-filtration and 5 µCi of the purified label was injected into the left a. carotis interna of 5 groups of 2-week-old chickens with 5 birds in each group (for anaesthesia see experiment A above). One group of birds was sacrificed immediately after the injection (1 sec) by suddenly decapitating the chickens and deep-freezing the opened skull on dry-ice-acetone. Birds of subsequent groups were sacrificed 5, 8, 12 and 16 sec after the injection of labelled albumin. This procedure was timed with high accuracy by the co-operation of two persons. One hundred to 500 mg portions of the ho-

mogenized brain were measured for ^{131}I activity in a well-type gamma counter (NZ-225, Gamma Works, Budapest, Hungary). Radioactivity was plotted against time as % of dose of the radioactivity. Selected samples were further processed to establish the leakage of ^{131}I from the albumin. Samples were chromatographed on Sephadex 10 columns.

Experiment C: Direct control of the 'single-pass' technique was carried out using anaesthetised (see earlier) birds from the same set of animals and injecting Iopamiro-370, a widely used radiographic contrast agent into the left a. carotis interna. Birds were placed under an X-ray machine and a series of shots were taken at 3-sec intervals.

Experiment D: Samples for the semiquantitative RT-PCR were obtained as follows. Thyroidectomy, sacrificing and the determination of thyroid hormones in the sera by radioimmunoassay were timed and carried out as mentioned above. The telencephalons of hypothyroid (TX) and normothyroid control (C) chickens were quickly removed and deep-frozen. Total RNA was isolated from the telencephalons by the method of Chomczynski (Chomczynski and Sacchi, 1987). cDNA was synthesised from 4 μg total RNA by Superscript™ II reverse transcriptase (Gibco BRL) according to the instructions of the manufacturers, using 1 μl (500 μg/ml) *Not* I-d$(T)_{18}$ primer (Pharmacia Biotech) per reaction.

cDNAs were standardised using a quantitative RT-PCR based approach for β-actin. Namely, a 741 bp fragment was amplified from cDNAs of the TX and C groups against a dilution series of a mutant plasmid, containing a 444 bp long, truncated β-actin fragment (kindly provided by Dr. I. Kacskovics) with the same set of primers. On the basis of the obtained data samples were diluted, to make them produce the same quantity of β-actin product. [Primers: sense = 5' ATG GAG AAG ATC TGG CAC CA 3'; antisense = 5' TCT TGA TCT TCA TGG TGC T 3'; 15 pM each. Cycles: 94 °C 2 min, (94 °C 20 s, 55 °C 30 s, 72 °C 90 s) 35 ×, 72 °C 4 min].

For the identification of thyroid hormone receptors, cDNAs were used after the β-actin standardisation mentioned above. The receptor amplification was stopped in the exponential phase of the reaction, in order to avoid reaching the plateau phase that could hide the expression differences via equalisation. The intensity of the bands between TX and C was compared both after TRα- and TRβ amplification. Each reaction was repeated at least three times. Primers were designed for sequences of lower homology and elevated annealing temperature was used to ensure receptor-type specific amplification.

TRα RT-PCR: sense = 5' GAG GCG CAC CGC AGC ACC AAC 3'; antisense = 5' ACT CGC TGA ACG CCT CCA GGT C 3'; 6 pM each; expected size of the product: 135 bp. (Sap et al., 1986). Cycles: 94 °C 4 min, (93 °C 30 s, 70 °C 1 min, 72 °C 15 s) 30 ×, 72 °C 5 min.

TRβ RT-PCR: sense = 5' CCG TGG CAG TGG TGA CAG AA 3', antisense = 5' CCG CCA TCT CCC CAT TTA GC 3'; 20 pM each, expected size of the product: 790 bp. (Forrest et al., 1990). Cycles: 94 °C 4 min, (94 °C 40 s, 65 °C 1 min, 72 °C 50 s) 28 ×, 72 °C 5 min.

Common PCR conditions: 50 mM KCl, 10 mM Tris HCl (PH 9.0 at 25 °C), 0.1% Triton® X-100 (dilution of 10 × reaction buffer), 1.5 mM $MgCl_2$, 133 μM dNTP (for each nucleotide), Taq polymerase 1 U/15 μl PCR end-volume, 1 μl first strand cDNA/15 μl PCR end-volume. (All reagents were supplied by Promega).

PCR products were run in 1% (for β-actin and TRβ) and in 2.5% (for TRα) agarose gels (FMC), stained by ethidium bromide. As a molecular marker, the 1 kb DNA ladder (Gibco BRL) was used.

All animal experiments were carried out with the consent of the Animal Experiments Board of the University of Veterinary Science, Budapest.

Results

Experiment A

The brain of the thyroidectomized (TX) birds showed a significantly higher uptake of ^{125}I-T_3 than in T_3 supplemented (TX + T_3) or sham-operated (SH) ones. The uptake was the highest in the telencephalons, while the cerebellum showed the lowest activity in this respect. The uptake was predominantly concentrated in case of the telencephalons to the left one which was the side of the injection. However, the differences between the uptake characteristics of TX, SH and TX + T_3 groups were maintained in the right hemisphere as well. Data are summarized in Table 1.

Table 1

Per cent of dose of injected ^{125}I-T_3 present in the brain homogenate of 2-week-old chickens 10 sec after a bolus injection of 5 μCi purified ^{125}I-T_3 with 0.02 g/BSA in 100 μl of saline. (Experiment A)

	TX	SH	TX + T_3
TEL right	0.16 ± 0.02 (9)[a]	0.09 ± 0.03 (10)	0.10 ± 0.02 (9)
TEL left	16.21 ± 1.22 (10)[b]	5.14 ± 0.87 (10)	6.28 ± 1.01 (9)
B + M	11.21 ± 1.21 (8)[b]	3.12 ± 0.47 (10)	4.13 ± 0.51 (8)
CM	6.14 ± 0.76 (9)[b]	2.68 ± 0.32 (10)	2.87 ± 0.49 (8)

TX = one week after surgical thyroidectomy; SH = sham-operated controls; TX + T_3 = surgically thyroidectomized + 3,3',5-triiodothyronine supplemented controls; TEL = telencephalon right or left side; B + M = brain stem + mesencephalon; CM = cerebellum. Superscripts [a] and [b] represent statistically significant differences at $P < 0.05$ and 0.01, respectively (TX vs. SH or TX + T_3). Average ± SEM (N)

Experiment B

Results of the ^{131}I-BSA injection showed that nearly 90% of the injected dose could be found within the brain 1 sec after the bolus injection which then rapidly decreased. This means that at the time of the decapitations in experiments A (10 sec after hormone injections) only around 3% of the ^{131}I-BSA was found in the brain. Later on the rate of disappearance decreased. The results of experiment B are summarized in Table 2. No detectable ^{131}I leakage could be demonstrated by chromatography.

Table 2

Per cent of dose of injected ^{131}I-BSA (bovine serum albumin) present in the total brain homogenate of 2-week-old chickens 1, 5, 8, 12 and 16 sec after a bolus injection of 5 μCi purified ^{131}I-BSA into the left arteria carotis interna. (Experiment B)

Seconds after injection of bolus	TX	SH	TX + T_3
1	93.97 ± 5.01 (5) ^{N}	91.27 ± 6.65 (5)	89.91 ± 6.18 (5)
5	6.71 ± 1.42 (5) ^{N}	7.14 ± 0.87 (5)	6.41 ± 1.01 (5)
8	3.21 ± 0.96 (5) ^{N}	3.37 ± 0.77 (5)	4.07 ± 0.88 (5)
12	1.86 ± 0.32 (5) ^{N}	1.67 ± 0.21 (5)	1.97 ± 0.76 (5)
16	1.57 ± 0.27 (5) ^{N}	1.45 ± 0.0.17 (5)	1.63 ± 0.23 (5)

TX = one week after surgical thyroidectomy; SH = sham-operated controls; TX + T_3 = surgically thyroidectomized + 3,3',5-triiodothyronine supplemented controls; Superscript ^{N} represents that no statistically significant difference at $P < 0.05$ was found (TX vs. SH or TX + T_3). Average ± SEM (N).

Experiment C

Two typical X-ray pictures are shown here demonstrating the uptake of the radiographic contrast agent, Iopamiro-370 into the brain. One can see that the skull of the experimental animal displayed high contrast at the left side of the head at the 15th sec after injection (Fig. 1). At 60 sec, however, uniform contrast shows an even distribution of the radiographic agent (Fig. 2).

These pictures prove that the time of sampling (10 sec after injecting the label) in experiment A is well before the re-entry of the label from the peripheral circulation. This demonstrates that the 'single-pass' nature of the experiment is substantiated.

Experiment D

The RT-PCR amplification of thyroid hormone receptors using β-actin standardised cDNA templates (Fig. 3) produced a markedly higher amount of product from cDNAs of the hypothyroid telencephalons than from that of the control both in case of TRα (Fig. 4) and TRβ (Fig. 5) specific reactions.

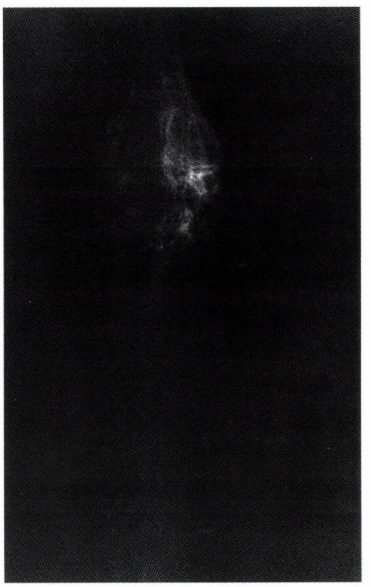

Fig. 1. X-ray picture at the 15th sec after injection of a radiographic contrast agent, Iopamiro-370 into the left arteria carotis interna of a 2-week-old chicken. The skull displays high contrast only at the left side of the head (experiment C)



Fig. 2. X-ray picture at the 60th sec after injection of Iopamiro-370 into the left arteria carotis interna of a 2-week-old chicken. Uniform contrast shows an even distribution of the radiographic agent (experiment C)

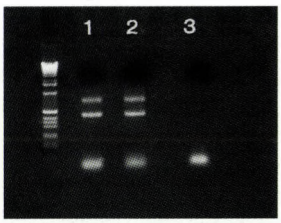

Fig. 3. β-actin RT-PCR after standardisation of cDNAs (see Materials and methods) synthesised from the telencephalon of control (1) and hypothyroid (2) 2-week-old chickens /neg. control: (3) Control and hypothyroid samples produced the same amount of 741 bp β-actin specific product against the same dilution of a plasmid, containing a 444 bp long, truncated β-actin fragment (lower band). [Marker: 1kb DNA ladder (Gibco BRL)] (experiment D)

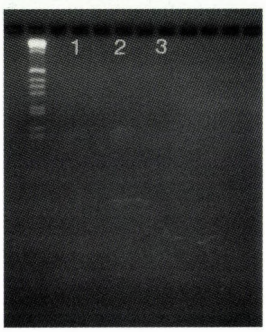

Fig. 4. Comparison of the expression of thyroid receptor TRα in the telencephalon of control (1) and hypothyroid (2) 2-week-old chickens by semiquantitative RT-PCR using β-actin standardised cDNAs [neg. control: (3)]. Reaction was stopped in the exponential-phase. [Marker: 1kb DNA ladder (Gibco BRL)]. The hypothyroid sample produced a higher amount of the 135 bp TRα specific product (experiment D)

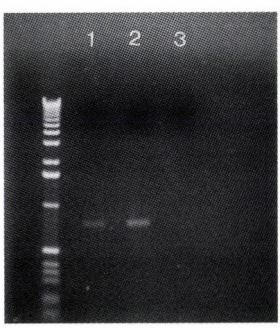

Fig. 5. Comparison of the expression of thyroid receptor TRβ in the telencephalon of control (1) and hypothyroid (2) 2-week-old chickens by semiquantitative RT-PCR using β-actin standardised cDNAs [neg. control: (3)]. Reaction was stopped in the exponential-phase. [Marker: 1kb DNA ladder (Gibco BRL)]. The hypothyroid sample produced a higher amount of the 790 bp TRβ specific product (experiment D)

Discussion

Experiment A strengthens our former findings (Rudas et al., 1993) that using Oldendorff's 'single-pass' technique one can demonstrate a considerable increase of the uptake of 3,3',5-triiodothyronine into the brain of hypothyroid chickens. The increased uptake may well be due to a possibly increased blood/tissue gradient for T_3 in the hypothyroid animals. This however seems not to be the case if we consider Silva and Mathews' (1984) and our former (Rudas, 1986) findings that in contrast to other tissues the brain preserves its T_3 level in hypothyroid animals for several weeks. Knowing this one can postulate that the increased uptake of the labelled T_3 is not the result of increased diffusion rate of the label according to its concentration gradient, but some specific blood-brain barrier transport mechanism should also exist. Such mechanism was found in the chicken by *in vivo* competitive methods (Rudas and Bartha, 1993). A study in mammals strengthens this view: the uptake of thyroxine into the brain of 10-day-old rat is three times higher than 20 days later, however the thyroid hormone content on day 10 is 5 times that on day 30 (Vigoroux et al., 1979).

The weakness of the single-pass technique is that one has to ensure an accurate timing in order to avoid the misleading effects of recirculation of the label (Pardridge, 1981). In this study it was proved in two distinct ways that under our experimental circumstances the 10th sec after injection of the label clearly satisfied that demand. Both the direct measurement of the fate of the bolus with radiographic contrast agents (experiment C) and the uptake of non-dialysable ^{131}I-BSA (experiment B) showed that at the 10th sec a maximum intracerebral amount of the bolus may be presumed and that no recirculation is encountered at this time point. Even if there is no recirculation at the time of sampling we have seen a detectable amount of labelled T_3 not only on the side of injection, but — although in negligible amounts — also on the contralateral side (Table 1, TEL right). This seemingly contradictory finding is not due to recirculation, however. It may be explained by the very tiny collateral vascularisation of the chicken brain that extends from the left a. carotis interna towards the right side. The presence of collaterals could be traced down by doing a caoutchouc model of several chicken vessel trees (Rudas and Bartha, 1993). The positive side of this small leakage to the contralateral side is that the increased uptake of the label was maintained also here in the case of TX animals.

Hypothyroidism-driven enhancement of the uptake of T_3 into the brain tissue prompted us to further investigate the intracellular events which could be involved in the adaptation of the brain to the altered thyroid status at the molecular level. The role of the deiodination enzyme system in the defence of the brain against hypothyroidism has already been proved (rat: Leonard et al., 1981; chicken: Rudas et al., 1993). However, another potent candidate is the nuclear

receptor system, since it was shown that the occupancy of thyroid receptors essentially influence the biological activity of thyroid hormones (Larsen et al., 1981), playing a crucial role in translating the intracellular thyroid hormone signal into biological answer.

Experiment D shows that hypothyroidism induced an elevation in mRNA abundance, coding the chicken TRα and TRβ receptors in the telencephalon. In addition, a line of data came from studies on the rat GH_3 pituitary cell line. A strong reduction of the pituitary-specific $TR\beta_2$ mRNA could be achieved by T_3 addition to the cultures but $TR\beta_1$ mRNA showed a slight increase after treatment (Hodin et al., 1989). $TR\alpha_1$ and $TR\alpha_2$ also responded via down-regulation to T_3 exposure in these cells (Lazar and Chin, 1988). This is in accordance with the earlier nuclear binding studies, showing a reduction of the binding capacity after T_3 addition in GH_1 cultures (Samuels et al., 1976). In contrast, expression of the TRα and TRβ mRNAs was unaltered by T_3 treatment in the brain of the rat (Hodin et al., 1990). In addition, it was observed in the liver of the rat that the reduction of $TR\alpha_1$ and $TR\alpha_2$ mRNA after T_3 administration was not followed by the fall of nuclear binding capacity (Strait et al., 1990). The interpretation of TR mRNA expression data as for its biological relevance should be handled with care, though. Especially in view of the findings that a remarkable thyroid-status related alteration of TR coding mRNAs was observed parallel with a relatively constant number of the receptor protein in the rat (Brent et al., 1991). There are at least three possible explanations for a discrepancy between the change of the amount of TR coding mRNA and the changes of the receptor protein itself.

First, it is possible that the investigated receptor type is not the physiologically important one in the given tissue due its relatively low presence, as it can be proposed in case of the TRα receptors of the liver (Strait et al., 1990; Schwartz et al., 1992). In contrast, our present study in chicken telencephalon showed the same phenomenon for both known receptor types. Interestingly, developmentally controlled huge elevation of the chicken TRβ receptor mRNA in the brain after embryonic day 19 (Forrest et al., 1990; Forrest et al., 1991) seems not to be followed by an increase in binding capacity, since the latter reaches its highest level on embryonic day 13 (Haidar and Sarkar, 1984).

Second, it is also suggested that an elevated mRNA level could be caused via mRNA stabilisation by the hormone itself, without increasing the transcription activity (Narayan and Towle, 1985). However, this phenomenon does not seem to be important in our case, since the study was made under hormone deficient conditions without exogenous hormone intake.

Third, the relevance of (post-)translational mechanisms was proved by investigating the early development of chicken erythroid cells, where the increase

of TRα protein was observed parallel with an essentially constant TR mRNA level (Bigler and Eisenmann, 1988).

Our present kinetics and TR expression studies are in line with our previous and other authors' findings, revealing the autonomous thyroid hormone handling of the central nervous system. To answer the question whether receptor up-regulation could be a tool for the brain of chicken during the adaptation to hypothyroid conditions, it seems to be necessary to complement the presented expression data by investigating the (post-)translational regulation of the TR proteins as well. It could be carried out by a comparative thyroid receptor binding study for the normo- and hypothyroid chicken brain which to our knowledge does not exist at present.

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ENDOSCOPIC METHODS IN SWINE REPRODUCTIVE RESEARCH: A REVIEW

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One of the major objectives of applying clinical methods is to get a more profound knowledge of the state of an organism. With the expansion of knowledge in physiology as well as the perfection of techniques the different applications have also improved. Over the past decade it has become obvious that in swine reproductive research such techniques are needed which do not influence the physiological processes and guarantee animal welfare. The minimal invasive and non-invasive methods (transabdominal endoscopy and ultrasonography) developed in human medical practice can be successfully adapted to the requirements of swine reproductive research. The present paper reports on the experience gained with abdominal endoscopy in swine so far.

Key words: Endoscopy, reproduction, diagnostic, surgery, swine

All optical diagnostic methods making possible the study of different ventricles without opening and exposing them to the outside environment are called endoscopy. According to the examined ventricle, e.g. abdomen and pelvis, the type of endoscopy is called laparoscopy and pelviscopy, respectively.

The first attempt made at examining ventricles was attributed to Bozzini (1805), who used a tube, a mirror and candlelight to observe the urinary bladder. The term 'endoscopy' dates back to the last century, when a French physician called Desormeaux used the name 'endoscope' to designate an instrument consisting of a petroleum light source and a mirror (cited by Benedict, 1951).

The introduction of modern gynaecological endoscopy (pelviscopy) is attributed to Semm, who indicated the advantages of the method in gynaecological and surgical practice and diagnosis (Semm, 1969; Semm and O'Neil-Freys, 1989).

Endoscopy in veterinary practice and animal physiology does not have such a long history as it has in medical research. In the 'seventies laparoscopy was done mainly in primates to master its use for human medical purposes, e.g. endoscopic biopsy (Dukelow et al., 1971; Jewett and Dukelow, 1972) and oocyte collection from squirrel monkeys for *in vitro* fertilisation (IVF) programs (Jewett and Dukelow, 1973). Dukelow and Ariga (1976) described surgical

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methods for laparoscopy in 23 species. They succeeded in blastocyst recovery from monkey uterus (Ariga and Dukelow, 1977).

Endoscopy is used widely in ruminants due to the uniparity of these animals. Endoscopic examination of the uterus and puncture of follicles in sheep were described more than 20 years ago (Snyder and Dukelow, 1974). Ovarian changes during the oestrous cycle of ewes were documented by daily laparoscopic observations (Oldham and Lindsay, 1980). The method of endoscopic flushing of ovine embryos was described in 1986. That procedure enabled the use of valuable donors much more times than would have been possible by laparotomy (McKelvey and Robinson, 1986). Endoscopic collection of tubal and uterine stage sheep embryos has also been reported (Kühholzer et al., 1996). The importance of that study is that the flushing of oviduct and uterus can be combined.

The biotechnological manipulation of early stage embryos was supported by laparoscopic tubal transfer in sheep as well as in goats (Besenfelder et al., 1994*a*; Besenfelder et al., 1994*b*).

In contrast to small ruminants, paralumbar endoscopy is used in the bovine (Chaffaux et al., 1980; Lambert et al., 1982; Bernard et al., 1984). Calves were born in IVF experiments following laparoscopy-guided oocyte aspiration (Sirard and Lambert, 1986).

Only few data are available on endoscopy in swine. Wildt et al. (1973) described laparoscopic examination of the reproductive organs without any surgical trauma and postoperative complication. Endoscopy was used to diagnose pregnancy as well as to collect uterine fluid during pregnancy (Wildt et al., 1975). Andersson and Einarsson (1980) compared data of hormone levels and endoscopic findings of the uterus in gilts during the first six oestrous cycles.

The oestrous cycle of miniature pigs was studied by laparoscopy repeated five times (Howard et al., 1982).

From studies on the influence of laparoscopy on follicular maturation, ovulation and reproductive function it was concluded that intensive and tough grasping of the ovary decreased the ovulation rate (Meisterling et al., 1984, 1985). Laparoscopy has been used for testing the consequences of ovario-hysterectomy and subsequent ovarian compensation (Christenson et al., 1987) as well as follicular development and homogeneity of follicles in different strains (Dufour et al., 1988; Grasso et al., 1988).

In earlier studies, optics and/or manipulator forceps were positioned laterally along the linea alba.

Later, it became evident that it is better for the large trocar containing the optic as well as for the small trocar introducing pliers to be pushed into the abdomen along the linea alba on the median line. It is better to push the optic directly behind the umbilicus and for the manipulator to insert between the last paps (Rátky and Wekerle, 1988). It is also evident that an experienced examiner can perform

diagnostic interventions by laparoscopy in 15–20 min. This speed makes the method suitable for field application, e.g. for revealing causes of infertility in pig farms (Wekerle et al., 1990; Rátky, 1993). In many cases this technique revealed the misdiagnosis of anoestrus instead of perfect oestrus detection. It was also demonstrated that laparoscopy could be repeated several times within short intervals (even within 20–30 min) on the same animal, enabling the follow-up of physiological events such as ovulation (Brüssow et al., 1990*a*).

Using this method, the morphological and dynamic effects of different treatments on physiological parameters, e.g. the relationship between the length of ovulation and exogenous GnRH induced LH-wave (Brüssow et al., 1990*b*; Brüssow et al., 1993) or between the length of ovulation and intravaginal infusion of seminal plasma (Brüssow and Rátky, 1993) can be monitored. The effect of long-acting GnRH-agonist on follicle maturation was also studied by endoscopy, and it was pointed out that during this treatment the follicles did not develop until the Graafian stage (Brüssow et al., 1994). The study of ovarian changes during the oestrous cycle was another example indicating the repeatability of the method. In that case laparoscopy was performed seven times before ovulation and during the luteal phase, then six times during the follicular phase (Rátky and Brüssow, 1992; Rátky et al., 1995).

After a 10-year-long practice and as many as 1400 diagnostic laparoscopies, we attempted to collect follicular fluid and to harvest oocytes by laparoscopy. The oocyte recovery rate reached about 55% by aspirating on day 17–18 of the cycle, while approaching the time of ovulation the collection rate of repeated aspiration on days 19–20 increased significantly to 70% and 69.1%, respectively (Brüssow and Rátky, 1994). These results demonstrate that repeated laparoscopy is a good tool for studying development *in vivo* as well as for collecting oocytes for IVF. A correlation was found between the recovery rate of cumulus-oocyte complexes (COCs) and the developmental stage of nuclei and that of follicles (Brüssow et al., 1995).

In another study, the degree of vacuum pressure on the rate of recovery and COC morphology was analysed, establishing the optimum pressure of 70–100 Hgmm (Brüssow et al., 1997).

Although embryo transfer is less important in pigs than in uniparous or safely cryopreservable species, it may be significant for some special research purposes. Utilisation of donor sows is limited because of laparotomy, so a new procedure was needed which would enable embryo collection three or four times per year. The results of ongoing research are promising: five-day-old embryos were flushed and a mean recovery rate of 40.4 % was achieved (Rátky and Brüssow, 1995). Good setting makes it possible to perform multiple surgeries and to produce embryos in a commercial quantity. The method has been introduced for

use in the propagation of endangered swine breeds in Hungary and so far more than forty piglets have been produced (Rátky et al., 1997).

Like similar techniques in small ruminants, tubal collection and transfer of porcine embryos have great importance in biotechnology, e.g. in gene transfer and cloning, and have been established recently (Besenfelder et al., 1997).

From the above it is evident that abdominal endoscopy has great potential in pig reproductive biology. By its use we can get such information on reproductive physiology which could not be obtained otherwise. This method allows the observation of reproductive organs without disturbing their integrity and reveals fine details. Being a 'non-invasive' technique, diagnostic laparoscopy can be repeated many times without disturbing physiology. By its repeatability, it allows us to follow-up the dynamics of physiological processes without slaughtering or sacrificing large numbers of animals at different intervals. Sampling by endoscopy at short intervals (1–2 days) helps us study *in vivo* changes in the same individual. Flushing embryos several times from donor sows is promising in research as well as in the breeding of top-quality or endangered animals. Its repeatability can be attributed to the fact that this method requires only minor invasion. The abdomen does not communicate with the outside environment, which is important for avoiding infection. No multiple cuts are made and, as compared with laparotomy, the field of vision is enlarged by the pneumoperitoneum. Postoperative recovery is faster, too.

The only drawback of the method is that it requires expensive equipment as well as a special surgical technique different from the traditional one, which requires a long period and great experience to master.

Nonetheless, endoscopy is an indispensable tool for research in reproductive biology.

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COMPARISON OF DIFFERENT TREATMENTS FOR PARTHENOGENETIC ACTIVATION OF BOVINE OOCYTES MATURED *IN VITRO*

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In vitro matured bovine oocytes were treated by three parthenogenetic activation methods. Basic treatment with Ca-ionophore and cytochalasin D was combined with cycloheximide (Group 2), electric impulse (Group 3), and cycloheximide and electric pulse (Group 1) treatments, respectively. Survival and the *in vitro* development of parthenogenetic embryos to hatched blastocyst stage were compared. Rates of the first cleavage and morula development significantly differed among the treatment groups. Further development rates of the treated embryos up to blastocyst stage did not differ. The best results were obtained by the combination of cycloheximide and electric treatments (Group 1; 14% blastocyst, 7% hatched blastocyst). The results suggest that the combined treatment for oocyte activation is the most efficient and should be applied for nuclear cloning procedures.

Keywords: Parthenogenesis, oocyte activation, bovine, *in vitro* produced embryos

Production of parthenogenetic mammals would be important in animal biotechnology and husbandry. It would allow the production of homozygous individuals within one generation and could be used to found inbred lines for heterosis breeding. Desirable alleles could be propagated in homozygous form that would have great importance in transgenic animal technology. It would make it possible to isolate valuable recessive mutations as well.

Parthenogenesis is a common phenomenon among insects, amphibians and reptiles. However, in mammals the parthenogenetically activated and implanted embryos are aborted in the intrauterine stage. In parthenogenetically activated oocytes further cleavage patterns follow those of the normally fertilised ones. Experiments with mice showed that the simultaneous presence and activity of the genetic material of both parents are essential for the birth of the progeny, while parthenogenetic animals abort in fetal life (Surani et al., 1984). Tissues of parthenogenetic origin could only be observed in chimeras produced by the aggregation of normally fertilised and parthenogenetic embryos (Nagy et al., 1989).

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There are different experimental methods to activate mammalian oocytes. They can be activated parthenogenetically by ethanol (Cuthbertson, 1983; Nagai, 1987; Páldi et al., 1989), Ca-ionophore A23187 (Ware et al., 1989; Shi et al., 1993), electric impulse (Ware et al., 1989; Yang and Smith, 1993), or by the combination of these methods (Fukui et al., 1992; Collas et al., 1993; Shi et al., 1993; Presicce and Yang, 1994; Du et al., 1995; Bodó et al., 1996; Soloy et al., 1997). These methods are based on two major mechanisms of the parthenogenetic activation: (1) the induction of a transient signal-like increase in the intracellular Ca^{2+}-level, simulating fertilisation by a spermatozoon, (2) the inhibition of the extrusion of the second polar body from the matured oocyte.

Cloning by nuclear transfer (NT) requires efficient activation of the recipient cytoplasm. Parthenogenetic activation experiments mainly contributed to the development of more efficient methods for NT, a method which, combined with somatic cell transgenesis and transfer, might be the key for a whole new era in animal breeding (Dohy, 1997; Solti, 1997). Nuclear transfer procedures are based on proper synchrony between the nuclear status of the fused blastomere and the activation status of the oocyte cytoplasm (Powell and Barnes, 1992). The optimal activation treatment for NT is the same as that for the production of parthenogenetic embryos (Du et al., 1995); therefore, parthenogenetic experiments can serve as control for NT. As the chromatin and cytoskeleton undergo profound changes during maturation, the success of NT is highly affected by the age of the oocyte. Nagai (1987) and Kubiak (1989) advised to activate the oocyte before the state of premature chromosome condensation (PCC), while others (Ware et al., 1989) reported activation of late-matured oocytes.

Our aim was to optimise the activation of matured bovine oocytes by studying the effects of different activation steps or their combinations in a parthenogenetic model system. The results of these experiments were successfully applied in our NT experiments (Dinnyés et al., 1997).

Materials and methods

Bovine oocytes were collected from slaughterhouse ovaries. After aspiration they were matured *in vitro* (IVM) in TCM 199 medium supplemented with 20% oestrous cow serum (OCS) (prepared in our laboratory) and 10 μg/ml FSH in 4-well dishes at 39 °C in a humidified atmosphere containing 5% CO_2 in air (Vajta et al., 1992). (All chemicals for experiments were purchased from Sigma Chemical Co., St. Louis, MO, USA.)

The cumulus cells were removed by vortexing in Ca^{2+}-free Dulbecco's modified phosphate buffer solution (DPBS) containing 0.2% hyaluronidase

23.5 h after the start of maturation. Oocytes selected for the presence of the first polar body (Fig. 2) were assigned to four treatment groups.

Group 1 oocytes were exposed for 5 min to 0.1 mM Ca-ionophore in phosphate buffered solution supplemented with 1 μg/ml polyvinyl alcohol at 24 h, then to 10 μg/ml cycloheximide (CHX) and 2.5 μg/ml cytochalasin D (CD) in TCM 199 with 10% OCS for 1 h. At 25 h cells were activated by an electric pulse (15 sec, 0.1 kV AC for alignment and 30 μsec, 1.2 kV DC impulse) in 0.3 M mannitol solution using an electro cell manipulator (ECM 200, BTX Inc., San Diego, CA, USA). Treated cells were incubated for 5 h in TCM 199 containing 10 μg/ml CHX, 2.5 μg/ml CD and 10% OCS. Between 30–48 h oocytes were incubated in 2.5 μg/ml CD in TCM 199 with 10% OCS, then transferred into a culture dish. The embryos were cultured *in vitro* (IVC) on granulosa monolayer in 4-well dishes in TCM 199 with 10% OCS at 39 °C in a humidified atmosphere containing 5% CO_2 for 10 days after activation until they reached the hatched blastocyst stage (Fig. 3). The timing of experiment is presented in Fig. 1 (Start of IVM = 0 h).

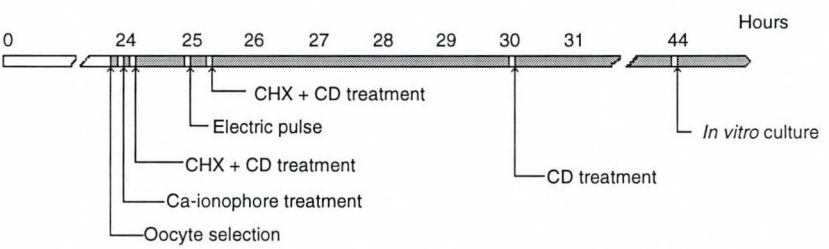
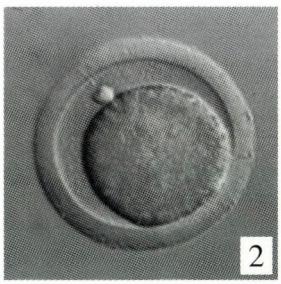


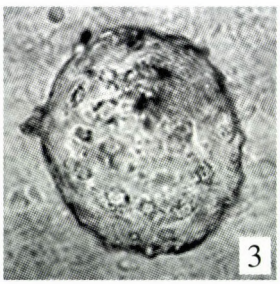
Fig. 1. Timetable for the parthenogenetic activation treatment of matured bovine oocytes

Group 2 and Group 3 oocytes were treated as above, with the exception that the electric activation step in Group 2, and the CHX treatment in Group 3 were omitted.

Group 4 (control) oocytes were fertilised *in vitro* (IVF, Vajta et al., 1992) and cultured *in vitro* as above.

At the end of the IVC period (day 10) blastocyst morphology was studied by Nomarski optics in an Olympus IMT-2 inverted microscope, at 400 × magnification, then embryos were fixed by ethyl alcohol and stained with Hoechst 33342 fluorescent dye. The number of nuclei was examined by an Olympus BH2-RFCA fluorescent microscope with UG-1 filter (Fig. 4). Data were analysed by the Chi-square method.





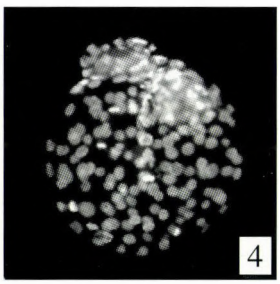


Fig. 2. Matured oocyte with polar body
Fig. 3. Hatched parthenogenetic blastocyst
Fig. 4. Nuclei of a hatched parthenogenetic blastocyst stained with Hoechst 33342 dye

Results

A total of 1475 bovine oocytes were collected and matured *in vitro*. The maturation rate based on the appearance of the first polar body was 49.9% (737/1477). A total of 675 polar body selected, good morphology oocytes were used for the activation experiments. Development of the embryos is presented in Table 1.

Table 1

Parthenogenetic development of bovine embryos following activation treatment

	Treated oocytes (n)	Cleaved zygotes[a] (%)	Morulae[a] (%)	Blastocysts[a] (%)	Hatched blastocysts[a] (%)
Group 1	163	47^{bc}	28^{b}	14	7
Group 2	174	33^{cd}	18^{c}	7	3
Group 3	159	38^{d}	18^{c}	8	3
Group 4	179	54^{b}	32^{d}	15	8

[a]rates of development in the percentage of treated oocytes. Groups with different superscripts (b, c, d) within the same columns are significantly different ($P < 0.05$)

Statistically significant differences were detected among groups in rates of early cleavage and morula development, but the differences were not significant in later stages. Group 1 (combined activation treatment) had similar hatched blastocyst yield to that of the control (Group 4).

Light microscopy revealed no obvious morphological differences between parthenogenetically produced embryos and controls. There was no significant difference in the cell numbers of parthenogenetically activated blastocysts compared to the controls (in average 120 vs. 128 cells/embryo, respectively) according to the fluorescent staining.

This database was analysed further to examine if the treatments had affected subsequent cleavage, therefore cleavage data were expressed in the percentage of the data of prior stages (Table 2). If the treatment had had any effect on subsequent cleavage, we should have obtained significant differences in the developmental competence of the embryos.

Table 2

Parthenogenetic developmental rates in percentage of the previous cleavage stage

	Cleaved zygotes[a] (%)	Morulae[b] (%)	Blastocysts[c] (%)	Hatched blastocysts[d] (%)
Group 1	47	59	50	48
Group 2	33	55	40	45
Group 3	38	49	42	32
Group 4	54	60	47	53

[a]cleavage rate in percentage of treated oocytes; [b]cleavage rate in percentage of cleaved zygotes; [c]cleavage rate in percentage of morulae; [d]cleavage rate in percentage of blastocysts

We could study the developmental competence of embryos in different treatment groups in each cleavage stage. The different comparisons show that the treatments affected significantly the first cleavage rates and the development to morulae (Table 1) but not the further developmental rates (Table 2).

Discussion

Matured mammalian oocytes are arrested in metaphase II. The activity of the cytostatic factor maintains a high level of maturation promoting factor (MPF) in the oocyte that is responsible for the arrest. The activity of both factors sharply decline after fertilisation or parthenogenetic activation. During *in vitro* maturation, ageing oocytes undergo a spontaneous parthenogenetic activation resulting in a low rate of parthenogenotes (7.5% of aged oocytes). Those parthenogenotes did not develop to hatched blastocyst stage (Plante and King, 1996). In order to induce further development, further activation effects are needed.

During mammalian fertilisation, the penetrating spermatozoon triggers a series of intracellular Ca^{2+} oscillations which initiate oocyte activation and the formation of pronuclei. Oocyte activation can be induced artificially by a variety of chemical and physical stimuli which elevate the intracellular calcium. In our experiments Ca-ionophore, electric pulse, and cycloheximide were applied for that purpose.

Oocytes must be in the proper stage to give an adequate reaction to the activation treatments. The meiotic resumption of the oocytes is determined by the level of MPF. As the MPF activity in oocytes requires protein synthesis, inhibitors of that process, e.g. cycloheximide, can be applied to suppress MPF activity. Arrest of the extrusion of the second polar body by the cytoskeletal inhibitor cytochalasin D mostly results in diploid parthenogenotes.

The application of CHX not only results in the depletion of proteins maintaining the MPF activity, but also prohibits the translation of cytoplasmic proteins controlling the initiation of DNA replication. The dual effects of CHX are the synchronisation of the pronuclear and the cytoplasmic compartments of the oocyte (Soloy et al., 1997).

Ca-ionophore treatment stimulates and synchronises the S-phase of the first cell cycle. When the parthenogenetic stimulus is an electric pulse, the S-phase is less synchronised and the electric activation reduces the efficiency of DNA synthesis. In the bovine, the electric activation alone did not result in hatched blastocyst development, and only 4% morulae/blastocyst have been produced by Yang and Smith (1993). These results suggest that a combined activation protocol, including Ca-ionophore which synchronises the cell cycles but does not damage the DNA synthesis, is beneficial.

Presicce and Yang (1994) used the combination of ethanol and CHX for activation. That treatment resulted in 32–44% cleavage, which is similar to the rates in our experiments (33–47%). Fukui et al. (1992) obtained 22–46% first cleavage and 5–11% blastocysts with a combined ethanol and cytochalasin-B treatment.

We studied the synergetic effect of activator chemicals and/or electric activation in different combinations based on the findings of Shi et al. (1993; Ca ionophore and CHX) and Yang et al. (1994; ethanol and CHX). Electric or CHX treatments alone resulted in cleavage rates lower than their combination. The combined treatment, when cycloheximide, cytochalasin D and electric pulse were applied, resulted in blastocyst development rates statistically not different from the fertilised controls. The first cleavage and morula development rates were significantly affected by the treatments. However, further blastocyst development was similar in each treatment group. The parthenogenetic activation treatments in our experiments did not cause significant difference in the results during the *in vitro* culture compared the control group. The developmental competence of the parthenogenetic embryos is similar to that of the *in vitro* produced embryos after the first cleavage.

Summarising the results, our findings suggest that supplementary electric activation besides the chemical treatments, probably due to an additive synergetic effect of CHX and electric impulse, is beneficial in increasing the activation rates and subsequent development. This treatment significantly affects the rate of first cleavage, but does not have much effect on later cleavages. Nuclear

cloning experiments based on our combined activation treatment resulted in improved cleavage and hatched blastocyst rates (Dinnyés et al., 1997), demonstrating the importance of an efficient activation method for cytoplasm donor oocytes.

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PROGESTERONE DETERMINATION IN EQUINE PLASMA USING DIFFERENT IMMUNOASSAYS

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Several assay systems [^{3}H radioimmunoassay (RIA) with and without extraction; microplate enzyme-linked immunoassay (ELISA); qualitative ELISA (tube test)] were used to measure plasma progesterone concentration in mare plasma. The direct RIA showed a close correlation (R = 0.94) with the extraction RIA. The direct RIA and the microplate ELISA were compared in two different studies. In the first study 1155 samples of postpartum mares were used for progesterone determination with both assays. The ELISA resulted in more elevated values both in oestrus and dioestrus (0.19 ± 0.3 and 2.44 ± 3.62 nmol/l for oestrus, n = 436, and 8.94 ± 4.29 and 27.88 ± 18.34 nmol/l for dioestrus, n = 719, for the RIA and ELISA, respectively, R = 0.71). The evaluation of individual progesterone profiles has revealed that the microplate ELISA detects the time of ovulation at the same time as it is determined by the RIA and clinical examination. The sensitivity and specificity were calculated for different progesterone threshold values. In the second study including 7 non-pregnant, cycling mares the progesterone concentration of 240 samples was determined by both assays. Basal values (Day 0) obtained with the ELISA were higher (1.57 nmol/l) than those of the RIA (0.2 nmol/l). Both curves reached the same maximum concentration (12.11 and 12.45 nmol/l) 5 days after ovulation. The correlation between the RIA and ELISA values was high (R = 0.90). The tube test was compared to the microplate ELISA as reference using 576 plasma samples of 34 non-pregnant, non-cycling mares included in an ovulation induction study. Of these samples 118 had higher and 458 had lower values than 3.18 nmol/l. In most cases the tube test was in complete agreement with the microplate ELISA. The sensitivity, specificity, + predictive and – predictive values for the tube test were 79.7%, 95.4%, 81.7% and 94.8%, respectively.

Keywords: Mare, progesterone, RIA, ELISA, microplate, mare-side test

Plasma progesterone determination in mares has a long history. In the original report Short (1959) was able to demonstrate the presence of progesterone only in case of a fully developed corpus luteum owing to the low sensitivity of the method (chromatography). It was in the early 'seventies that more sensitive immunoassays (competitive protein binding assay and radioimmunoassay)

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were introduced to monitor plasma progesterone changes throughout the oestrous cycle (Smith et al., 1970; Plotka et al., 1972; Stabenfeldt et al., 1972). All these assays required some sample purification, usually extraction with petroleum ether.

Shortly after the introduction of these new techniques there were already attempts to simplify or replace them in order to have cheaper, faster and more practical means to determine plasma progesterone in domestic animals. In the first step the extraction procedure was skipped, but RIA was still used (Stupnicki, 1975; Mathieu et al., 1977; Srikandakumar et al., 1986). The recently developed quantitative ELISA systems (Munro and Stabenfeldt, 1984; Solti et al., 1984; Allen and Sanderson, 1987; Eckersall and Harvey, 1987; Solti et al., 1990; Lopate and Threlfall, 1991) may also provide good results with less time, cost and labour demand.

Since in most cases a Yes or No answer (progesterone present or not) is sufficient in equine gynaecology, the development of qualitative enzyme-linked immunoassays (mare-side tests) gave a powerful tool into the hand of clinicians. With such commercially available tests (Elmore et al., 1988; Hinrichs et al., 1988; Thompson and Bergfelt, 1990) mares can be followed in the spring transition period, ovulation can be confirmed, the background of anoestrus can be investigated, and pregnancy checks can also be performed (Allen and Sanderson, 1987; Elmore et al., 1988).

The aim of this study was to compare different immunoassays (direct and extraction ^{3}H-RIA, microplate and qualitative ELISA) developed in Hungary for the determination of progesterone in plasma of mares at various reproductive stages (postpartum, cyclic and anoestrous mares).

Materials and methods

Assay procedures

First, progesterone determination was carried out with liquid-phase ^{3}H-RIA with and without extraction. The details of the extraction assay were reported by Huszenicza et al. (1993*a*). In the two RIA systems the same polyclonal anti-progesterone antibody was used (Csernus, 1982). In the direct RIA 100 µl ^{3}H-labelled progesterone ([1,2,6,7-^{3}H] Progesterone, Amersham International, England; 12–15000 disintegration per minute) and 100 µl (1 : 17,500) antibody were put into assay tubes containing 20 µl of standards, unknown or control samples and 200 µl of assay buffer at 4 °C. The antibody-bound and free fractions were separated after 18 h of incubation at 4 °C by 300 µl of cold dextran-coated charcoal suspension and by centrifugation at 3000 rpm for 10 min. The supernatant of the assay tubes was poured into scintillation vials and 5 ml of

counting solution was also added to each vial before counting. The sensitivity of the assay was 17.6 fmol/tube (equal to 0.88 nmol/l progesterone in plasma). The intra- and inter-assay coefficient of variation for plasma pools representing low (3–4 nmol/l), medium (6–7 nmol/) and high (15–19 nmol/l) ranges were 15.4%, 7.6%, 8.0% and 13.0%, 9.7%, 7.3%, respectively.

The recently developed microplate ELISA used an anti-progesterone mouse monoclonal antibody (5D4; Siklódi et al., 1995). The assays were carried out at room temperature by adding 20 µl of sample (standard) and 180 µl of conjugate (horseradish peroxidase labelled progesterone) into each well (EDISTIR N 1841; Analyser, Hungary). After 50 min incubation the content of the wells was discarded and the plates were washed 4 times with washing solution. The enzyme-substrate reaction was initiated by adding 200 µl/well of freshly prepared substrate solution which contained tetramethylbenzidine (TMB) and H_2O_2 in citric phosphate buffer (pH = 5.5). The development of the blue colour was stopped after 5 to 15 min by 50 µl/well 6N sulphuric acid. The intra- and inter-assay coefficient of variation for plasma pools of 4–5 nmol/l were 10.6 and 13.1%, respectively.

The tube test (qualitative ELISA) used the same monoclonal antibody as the microplate ELISA. The assay was performed by the same person. Three hundred µl of sample was pipetted into an antibody-coated tube, followed by 1.2 ml of conjugate (solution A). At the same time a control sample containing 3.18 nmol/l progesterone was also assayed as reference. After 30 min all tubes were emptied and washed 5 times with cold running water and emptied again. In the meantime the substrate solution was prepared freshly by adding 1 drop of TMB solution (solution C) to each ml of citrate phosphate buffer and H_2O_2 (solution B). After adding 1.5 ml substrate solution the tubes were left for another 5 to 15 min to allow the colour change to develop. The colour intensity of the sample tubes was compared to that of the control. Results were reported as positive (high progesterone, no colour development or paler colour than that of the control), negative (low progesterone, blue colour darker than that of the control), and questionable (blue colour similar to that of the control).

Sample collection and assay comparison

For all studies, blood samples were collected by jugular venipuncture in heparinised tubes. Samples were either centrifuged for 10 min at 3000 rpm shortly after collection or left standing for 20 min before harvesting the plasma which was then stored at –20 °C until assayed.

Some samples of a previous seasonality study (Huszenicza et al., 1993*b*) were assayed by both the conventional extraction RIA and the direct method. The results were compared with linear regression.

The direct RIA and the microplate ELISA were compared in two different studies. In the first study 65 foaling mares were followed by three times a week blood sampling, regular teasing and ultrasonography from foaling till pregnancy. The details of this study will be published elsewhere. Data were compared by different methods. Not only linear regression of individual samples was performed but also entire progesterone profiles were analysed. RIA and ELISA profiles were evaluated for similarity both visually and using an algorithm. The algorithm was designed to find the most important changing points (oestrus – dioestrus) of a given profile. For each point of the curve it calculates the difference between the average of 3 samples after and 2 samples before that particular point. The place (time) of ovulation is put to the point where this difference is the greatest. An additional condition was also used in the algorithm to eliminate the detection of greater changes in the progesterone-plateau: in case of the RIA and the ELISA the average of the 2 samples before the point had to be below 3 and 10 nmol/l, respectively.

Samples were also classified according to reproductive status (oestrus, dioestrus) of the mares and the sensitivity and specificity of both assays were calculated at different progesterone levels as changing points. The sensitivity and the specificity are defined as $a/(a + d) \times 100$ and $c/(c + b) \times 100$, respectively, where a = correct positive, b = false positive, c = correct negative, d = false negative diagnosis.

Another opportunity to compare the direct RIA and the microplate ELISA was a study (Juhász et al., 1997) including non-pregnant, cycling mares in which daily progesterone was determined by both assays from the day of ovulation for the following 5 days. RIA and ELISA values and their difference was analysed with linear regression. Analysis of variance of the difference was performed with the day of cycle and the progesterone concentration as main effects.

The tube test was compared to the microplate ELISA in a seasonality study where the values obtained with the quantitative assay were used as reference. During that study 34 non-cyclic barren mares were followed by regular teasing, rectal examination and twice-a-week blood sampling at the beginning of the breeding season. Sensitivity, specificity, correct and false positive, correct and false negative diagnosis were determined based on agreement with the microplate ELISA.

Results

RIA with and without extraction

One hundred and two samples of 9 mares were assayed by both RIA systems. In 6 cases the difference in the values obtained was over 6 nmol/l. This difference occurred systematically in the higher range (P4 > 14 nmol/l) as de-

termined by the extraction RIA), which is due to the lower accuracy of the direct system at that concentration range. There is another systematic difference between the two assays on the other side of the standard curve which is related to sensitivity: in 39 cases progesterone was determined by the extraction assay (ranging from 0.1 to 0.9 nmol/l) whereas in the direct system these samples resulted in counts per minute over those of the maximum binding. Besides these values there is no systematic difference (R = 0.03), the average difference is 0.33 nmol/l (SD = ± 1.7 nmol/l) and the relationship is quite close between the values obtained in the two systems, as shown in Fig. 1 (R = 0.94, slope = 0.989, $P < 0.001$).

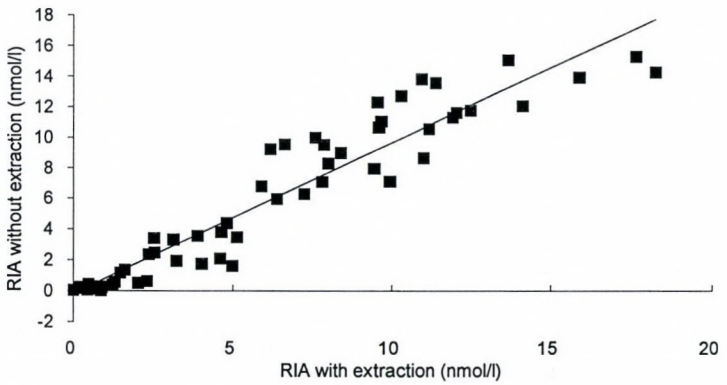

Fig. 1. Comparison of progesterone RIA with and without extraction. There was a close relationship (R = 0.94) between the extraction and the direct RIA

Direct RIA and microplate ELISA

The direct RIA and the microplate ELISA were compared in two different studies. In the first one including 65 foaling mares 1155 samples were tested by both assays to follow up postpartum ovarian activity. Owing to the cross-reactivity of the monoclonal antibody used for the ELISA with some of the progestins produced by the equine placenta only samples more than 10 days after foaling were used for the statistical analysis. Figure 2 shows the progesterone-profile of a mare. It can be seen that the main reproductive stages (oestrus, dioestrus) are well differentiated with both assays, although dioestrous values may differ significantly. In general, the ELISA resulted in more elevated values and this is the reason for the decreased correlation between the two methods (R = 0.71).

When entire progesterone-profiles were evaluated, it was concluded after visual evaluation that the microplate ELISA detects the time of ovulation in the same way as it is determined by the RIA and clinical examination. Since 'visual

evaluation' of the curve may not be precise enough in terms of statistics, an algorithm was also used to compare the two methods. Using this technique, the RIA detected 76 out of the 86 ovulations that occurred in the course of the study. In the case of 10 ovulatory periods the false negative result was related to the low number (3) of elevated progesterone values after ovulation. That was due to the short luteal phase (sampling interval: three times a week). Of the 76 points detected by the RIA 74 were also found by the ELISA and 69 were exactly at the same place. In the 5 remaining cases the algorithm found the point on the ELISA curve but it put it either before or after the place of the RIA. In two cases the program found no elevation on the ELISA curve as compared to the RIA.

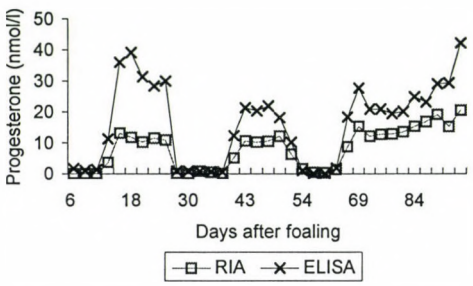

Fig. 2. Plasma progesterone-profile of a mare after foaling, determined by both the RIA and the ELISA. Despite the difference in dioestrous concentration both assays show the time of ovulation at the same time

At each sampling date all mares were classified to be either in oestrus or in dioestrus based on clinical data. It was found that the mean progesterone concentrations during oestrus were 0.19 (SD ± 0.3, n = 436) and 2.44 (SD ± 3.62) nmol/l and in dioestrus 8.94 (SD ± 4.29, n = 719) and 27.88 (SD ± 18.34) nmol/l for the RIA and ELISA, respectively.

Using different progesterone levels as threshold value for luteal activity the sensitivity and specificity were calculated for both assays (Figs 3 and 4). In the RIA the sensitivity (the ratio of true positive, dioestrous samples) decreases gradually from 100% to 38.4% as the progesterone threshold value increases from 0 to 10 nmol/l. In contrast, the specificity (the ratio of true negative, oestrous samples) increases sharply and reaches 100% at the 2 nmol/l level and remains the same afterwards. Both the sensitivity and the specificity are high at 1 and 2 nmol/l indicating the proper threshold range as the sign of dioestrus for the RIA. If the traditional 3.2 nmol/l (1 ng/ml) limit is to be used as the indicator of dioestrus there is approximately 8% decrease in sensitivity (increase in false negativity). In the ELISA the sensitivity decreases only slightly, from 100% to 90.3%. The specificity increases gradually from as low as 3.4% to 98.4%, from

0 to 10 nmol/l. For the ELISA higher values are to be used as borderline between oestrus and dioestrus since at the 2 nmol/l limit the specificity is much lower (69.4%, high proportion of false positive values) than that of the RIA. At 5 nmol/l both the sensitivity (97.75%) and the specificity (91.92%) are high.

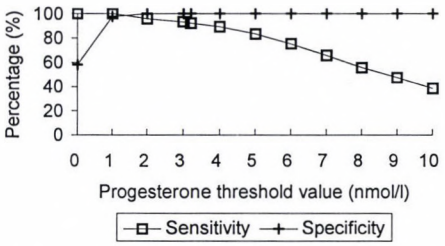

Fig. 3. Changes in sensitivity and specificity of the direct RIA in relation to different progesterone threshold values as a sign of luteal activity. In the direct RIA 1–2 nmol/l of progesterone is the proper threshold for corpus luteum function

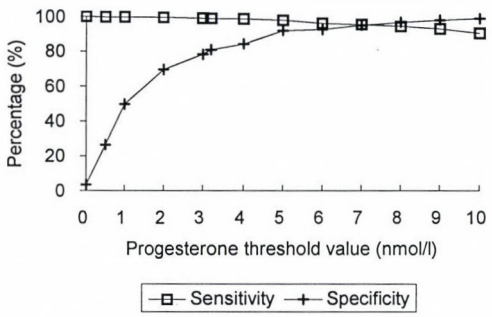

Fig. 4. Changes in sensitivity and specificity of the microplate ELISA in relation to different progesterone threshold values as a sign of luteal activity. In the ELISA the threshold for corpus luteum function should be set higher (3–5 nmol/l) than for the RIA

In the second study 240 samples of 41 ovulatory periods from day 0 (the day of ovulation) till day 5 were assayed by both the RIA and the ELISA. Changes in plasma progesterone after ovulation are shown in Fig. 5. It can be seen that basal values (day 0) obtained with the ELISA are higher (1.57 nmol/l) than those of the RIA (0.2 nmol/l) and both curves reach the same maximum concentration (12.11 and 12.45 nmol/l) 5 days after ovulation. The standard error of means varies between 0.06 and 0.8 nmol/l. The correlation between the RIA and ELISA values is fairly high ($R = 0.90$, slope = 0.89, $P < 0.001$) (Fig. 6).

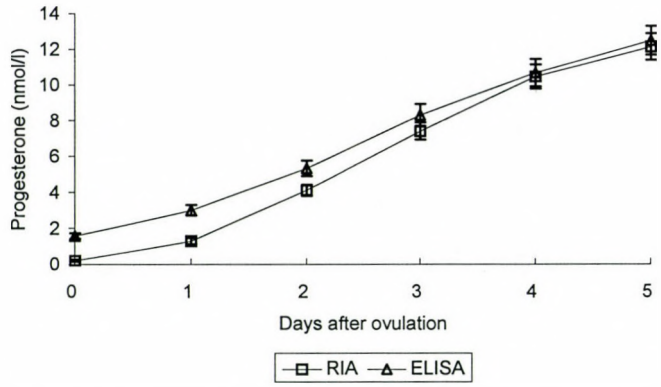

Fig. 5. Changes in plasma progesterone (mean) after ovulation as determined by both the direct RIA and the microplate ELISA. Error bars represent standard error of mean. Note the different oestrous (day 0) and the same dioestrous (day 5) concentrations

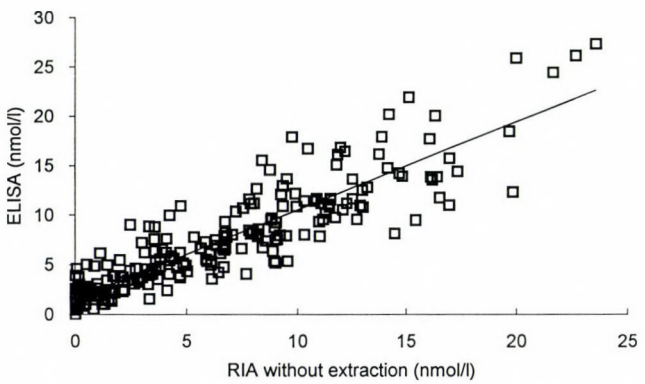

Fig. 6. Comparison of the direct RIA and the microplate ELISA values (second study). The correlation between the direct RIA and the microplate ELISA was fairly high (R = 0.90)

When the difference between the RIA and ELISA values was studied in relation to the time of ovulation and to RIA absolute values, it was found that in the higher concentration range (RIA > 10 nmol/l) there was no significant relationship between the RIA values and the difference from those of the ELISA (mean difference: –0.024 ± 3.36 nmol/l, $P < 0.970$). On the other hand, in the lower range the difference became significant (1.21 ± 1.925 nmol/l, $P < 0.0091$), reflecting a systematic difference in the low range between the RIA and the ELISA. The same trend was observed when the day of ovulation was used as classification. The analysis of variance by the day of ovulation and RIA values revealed that it was the effect of the progesterone concentration itself ($P < 0.001$) rather than that of the time after ovulation ($P < 0.827$).

Microplate ELISA and tube test

Five hundred and seventy-six serially collected samples of 34 mares in the spring transition were tested by both the tube test and the microplate ELISA. Of these plasma samples 118 were higher and 458 were lower than 3.18 nmol/l as determined by the quantitative ELISA. In most of the cases the tube test was in complete agreement with the microplate ELISA except 21 and 24 cases when the tube test resulted in false positive and false negative results, respectively. The sensitivity, specificity, the false positive and negative rates and the positive and negative predictive values of the tube test are shown in Table 1.

Table 1

The features of the diagnostic validity of the mare-side tube test for progesterone as compared to the microplate ELISA

Sensitivity (%)	79.66
Specificity (%)	95.41
False positive rate (%)	4.59
False negative rate (%)	20.34
+ Predictive value (%)	81.74
– Predictive value (%)	94.79

Discussion

The most important prerequisite of basic endocrinological investigations in mares is a precise and accurate assay for progesterone. A ^{3}H-RIA with extraction like the method used in our earlier works (Huszenicza et al., 1993*a, b*) fulfils these criteria but it also has a number of drawbacks. Sample purification makes the assay slow, expensive and decreases its output. On the top of that it is not suitable for field veterinary use. For this reason in the present study recently developed no-extraction RIA and ELISA assays were compared in the testing of mare plasma. Progesterone binding of equine plasma is weak, constant and non-specific (Mathieu et al., 1977). This is the reason why such no-direct-extraction immunoassays could be applied successfully in horses.

The first assay tested was a direct ^{3}H-RIA validated for equine plasma. The advantage of such direct RIA systems is that they are usually inexpensive, easy to perform in one or two days, and still maintain good validation parameters allowing their use for both clinical and research purposes (Srikandakumar et al., 1986). In our circumstances (considerable distance between the studs and the laboratory), despite some loss of sensitivity the assay was found to be suitable

for research but not for daily clinical use. During oestrus the extraction RIA was able to measure plasma progesterone whereas in some cases the direct assay resulted in calculated 0 nmol/l (counts per minute above that of the maximum binding). From the physiological point of view such a difference can be neglected since both results mean no luteal activity. The assay was quite accurate although at the higher part of the standard curve (>15 nmol/l) some decrease in accuracy was encountered. In addition to sensitivity, that was the reason for the slight difference between the direct and the extraction RIA which showed very good correlation (R = 0.94). This is in contrast to the results of Srikandakumar et al. (1986) who found much higher dioestrous values with the direct assay. In their case the extraction RIA was similar to ours but their direct system was a solid-phase ^{125}I-RIA with different antibody than that of the extraction RIA.

Even though our direct RIA has been considered to be very appropriate for research purposes, it has some disadvantages (time and laboratory requirement, radioactive and inflammable waste) which could be overcome by either the use of microplate ELISA or of a mare-side test. The enzyme immunoassay tested in our study is easy to perform, takes only 2 h at room temperature and does not require specific laboratory skills. Its most important advantages over the RIA are the relatively low costs (liquid scintillation counting for the RIA) and the potentially high daily sample output. The large number (approximately 1400) of samples that were assayed by both methods allowed us a precise evaluation of this technique. In the postpartum study it was convincingly demonstrated that individual RIA and ELISA plasma progesterone-profiles were similar. The profile-analysis was done not only visually but also using a non-parametric method. The microplate ELISA detected the oestrus and the dioestrus at the same time as the direct RIA. When absolute values were compared, it was found that during dioestrus important differences might occur between the two methods. The same tendency was also found by others (Eckersall and Harvey, 1987). This difference is mainly due to the different cross-reactivity of the two antibodies used in RIA and ELISA.

It was also noted that in the ELISA progesterone basal (oestrous) values were higher. From the diagnostic point of view this is more important than the dioestrous difference, since in practical terms only limited additional information could be obtained from the absolute value of the elevated progesterone level. On the other hand, if only one single sample is available to determine the reproductive status of a mare, it is extremely important to know the threshold value for luteal activity. This was demonstrated in Figs 3 and 4 by showing the changes in specificity and sensitivity in relation to different progesterone threshold values. The pattern of both the sensitivity and specificity curves is not similar, indicating the different features of the RIA and ELISA around the time of ovulation. Using the same threshold value for both assays mainly the specificity

(the ability of the assay to detect true negative, oestrous values) would show great difference. Two nmol/l plasma progesterone determined with the direct RIA clearly indicates luteal activity whereas the same value obtained by the microplate ELISA may only mean that the mare has no functional luteal tissue. Based on these, 1–2 nmol/l and 3–5 nmol/l plasma progesterone can be determined as a threshold for luteal activity for the direct RIA and the microplate ELISA, respectively.

Our finding concerning the different basal concentration was repeated in the second study including the barren mares. On the other hand, the average daily dioestrous values became increasingly similar and the two curves reached the same maximum concentration (Fig. 5). That was the reason for a much better correlation (R = 0.9) between the RIA and the ELISA values. It is not completely understood why the maximum dioestrous values obtained with the ELISA in the two studies are so much different. One of the reasons is that in the second study mares were sampled only for 5 days after ovulation whereas in the first one samples from pregnant mares were also assayed. The difference may also be attributed to the fact that the two studies were performed in different years and in different laboratories although the same material (except the conjugate) was applied.

Some of the disadvantages of the ELISA system is its decreased precision as compared to the RIA (Eckersall and Harvey, 1987). In order to maintain proper assay quality, low and high plasma pools were also included on all plates like in the RIA. Our intra-plate coefficient of variation was always close to the range of the RIA but sometimes unexpectedly high inter-plate variation was observed. Usually this did not occur on the same day and was most probably related to the time elapsed between antibody coating and the assay. In all cases when such a problem occurred the assay was repeated using a freshly prepared plate.

In the last decade there was a great demand from field veterinarians to introduce semiquantitative tests for progesterone which do not require laboratory facilities and can be carried out under farm conditions. Nowadays there are several tests available on the market but only a few studies comparing laboratory methods with mare-side tests have been published (Elmore et al., 1988; Hinrichs et al., 1988; Thompson and Bergfelt, 1990). The tube test used by us has already been proved to be suitable for bovine embryo transfer recipients (Solti et al., 1994). It takes only 40 to 50 min to run at room temperature and is quite easy to evaluate on the basis of colour changes. In the present study its usefulness was evaluated by comparing tube test results with those of the microplate ELISA. Approximately the same number of samples were incorrectly diagnosed as positive (above 3.18 nmol/l) and negative (below 3.18 nmol/l), but owing to the different number of low (n = 458) and high (n = 118) samples the specificity was more than 15% higher than the sensitivity. In addition to these parameters it is

more important to know the probability (+ and – predictive value) that the test is correct. It has been shown that the negative predictive value of the tube test is fairly high (94.8%) but the positive predictive value is not so good. In other words, the test is really suitable for monitoring the vernal transition till the first ovulation of the year (low false-positive rate) when none of the samples contain progesterone. On the other hand, if the background of anoestrus is to be investigated during the breeding season (cyclic ovarian activity is assumed) the chance that a dioestrous mare is misdiagnosed as being in oestrus (low progesterone) is higher.

Finally, it can be concluded that different assays are available for measuring plasma progesterone in horses. The method of choice depends on a number of factors such as availability, aim of the work, costs etc. When absolute values are important (like in a study on factors affecting circulating progesterone) the most accurate system (RIA) should be used. In cyclicity studies the microplate ELISA provides acceptable results and in the field the tube test may give an invaluable answer to the clinician.

Acknowledgements

We would like to thank the Hungarian Scientific Research Fund (OTKA) for financial support (project number: T 16473). The excellent technical assistance of Ms Erzsébet Kiss, Ms Ibolya Simon and Mr Béla Budai with the RIA and microplate ELISA and that of Attila Balogh with the tube test are gratefully appreciated.

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Acta Veterinaria Hungarica 46 (4), pp. 515–517 (1998)

BOOK REVIEWS

Uli Braun (ed.): ***Atlas und Lehrbuch der Ultraschalldiagnostik beim Rind.*** With the contribution of Mark FLÜCKIGER, Wolfgang KÄHN, Johann KOFLER, Christoph J. LISCHER, Peter RÜSCH, Adrian STEINER, Hans STOCKER. Parey Buchverlag, Berlin, 1997. 289 pages, 300 figures, and 75 tables. Price: DM 248. ISBN: 3-8263-3092-7.

Ultrasonography has been used as a conventional and feasible diagnostic modality both in man and animal species for several decades. In veterinary medicine, numerous publications and books deal with the application of this technique in companion animals. However, bovine ultrasonography was mainly confined to the ultrasound examination of the reproductive organs. The present work is the first textbook which describes the ultrasonographic findings of several organs and provides information on alterations of these organs in gynaecological, internal and surgical diseases of cattle.

The book is well-structured, written in a concise manner, and provides the reader with easy access to the information on different body systems and organs included in the well-illustrated chapters as follows: basic principles of ultrasonography, reticulum, liver, abomasum, intestine, pleura, lungs and mediastinum, urinary system, udder and teats, abdomen, abdominal cavity, uterus, fetus and ovaries, umbilicus, locomotor system.

Each chapter on the ultrasonography of a particular organ is structured in the same, clearly organised paragraphs, i.e. anatomical and physiological aspects, technique of examination, normal findings, ultrasonographic characteristics of pathological alterations, list of reference works. Several well-organised tables, high-quality ultrasound images, and corresponding schematic drawings are added to the text which are of great value for readers studying this work. Comparison of ultrasonography with radiography is discussed briefly, but no radiographic images are included. A few ultrasonographic alterations are also demonstrated with the help of simultaneous morphological pictures, which could have perhaps been applied more often. The book provides comprehensive knowledge both for the daily work of the ultrasonographer and for veterinary students as well as other veterinarians interested in this technique. However, one will be missing bovine echocardiography which has not been included in this textbook. This drawback may be corrected in the next edition. As no equivalent work on bovine ultrasonography is currently available, it would also be worth publishing an English edition in the near future.

Károly Vörös

Josef ASSHEUER and Martin SAGER: ***MRI and CT Atlas of the Dog.*** Blackwell Science Ltd., Oxford, 1997. 482 pages, with over 1200 illustrations. Price: DM 298. ISBN 3-89412-164-5.

Conventional radiographic techniques have been used by veterinarians for decades. Today, ultrasonography is routinely used also in the everyday veterinary practice. The new, two-dimensional topographical diagnostic imaging techniques, CT and MRI are just being introduced into veterinary medicine. The first edition of this atlas makes a significant contribution to this process.

The atlas presents the cross-sectional whole-body anatomy of the dog. Besides a short but clear introduction into the basic principles of these two imaging techniques — which for some reason can be found at the end of the book — the atlas is divided into 7 chapters, according to different body regions: head, spine, thorax, abdomen, pelvis, thoracic and pelvic limb. Particular attention is focused on the head and spine, as they almost fill half of the book. Each chapter starts with a short introduction about the anatomy, scan techniques and main pathological processes in the particular region. Examples of MR images of pathological changes are given herein. The imaging part of normal anatomy starts with CT images in transverse orientation that show the cross-sectional image of the given region, especially the calcium-containing bony parts in high detail. The MRI scans, which are placed to the right of the CT images with approximately the same slice position and orientation, show soft tissues in higher detail. Sagittal and dorsal MRI scans with different tissue contrast follow the transverse presentation of a body region. Anatomical structures on an image are marked with lines which are labelled using abbreviations. These abbreviations are listed in the legend in alphabetic order.

The aim of the authors was to provide reference cross-sectional anatomical information for practitioners and specialists. However, even veterinary students will find this book useful in understanding the topographic anatomy of the dog. The main advantage of the book is the simultaneous presentation of anatomical images and CT/MRI scans which serve as guidelines when performing these new imaging techniques.

Ferenc Manczur and Károly Vörös

Eurovet Guide/A Guide to Veterinary Europe 1998–1999. Les Éditions du Point Vétérinaire, France, 1998. 2nd edition. Editor: Karin de Lange, DVM, MRCVS. 680 pages. Price: 310 FF. ISBN 2-86326-141-X.

This second, completely updated edition of the Eurovet Guide (680 pages) contains over *6000 names and addresses*. Some *560 veterinary professional and specialist associations* (over 40 disciplines) from all over Europe are presented, with their current board members and contact addresses. The Eurovet Guide also gives an overview of veterinary *specialisation* today in the various countries and on European level.

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