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THE DISTRIBUTION AND STRUCTURE OF THE MICROVASCULATURE OF THE PHALLUS IN THE OSTRICH (*Struthio camelus*)

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ABSTRACT

This study determined the distribution and specialized morphology (gaps and fenestrations, irregular endothelium, internalization of luminal material) of vessels in the spongy and erectile tissue of the phallus in the ostrich. Specimens from the phallus harvested from 18 sexual mature and active ostriches were utilized. General routine fixation was used for tissue blocks and then they were processed conventionally for observation under the Transmission Electron Microscope (TEM). The spongy tissue in the root of the phallus exhibited several blood vessels (mainly venules) within connective tissue strands. The capillaries displayed thick and attenuated endothelial profiles, whereas the venules were lined with an attenuated endothelium. Both vessel types displayed fenestrations and prominent gaps supported by a continuation of the basal lamina. Numerous cytoplasmic processes extended from the capillary endothelium, in places appearing to enclose and sequestrate luminal contents by phagocytosis. The internalized material appeared in the form of relatively large, pale vesicles. The endothelium was extremely irregular in profile, in some instances appearing to be composed of more than one layer of squamous cells. The erectile tissue lining the phallic sulcus presented relatively few capillaries and venules. These vessels exhibited features similar to those seen in the spongy tissue but the endothelial cells were markedly more complex and irregular in design.

Key words: erection mechanism; male ostrich; microvasculature; phallus; reproductive system

INTRODUCTION

The phallus is a structure formed by closely related folds of tissue that derives from the ventral wall of the proctodeum in male avian species. It is analogous to the mammal penis, because it transfers ejaculated spermatozoa to the female reproductive organs [1]. There are two types of phalluses, the intromittent (penetrates the female cloaca) and non-intromittent (phallus does not penetrate the female cloaca, male and female cloaca are simple pressed

together to transfer sperm). The ostrich phallus is of intromittent type. The erection of the phallus was investigated by Rautenfeld [15], but this still needs more research using modern techniques [14].

The gross morphological structure of the ostrich phallus has been described [2, 5, 8]. No information is available on the microvascularization of that segment of the reproductive organs, although the gross pattern of both arterial supply and venous drainage of the male reproductive tract of the ostrich has been investigated [3, 4, 18]. The paracloacal vascular body (*corpus vasculare paracloacale*) is the source of lymph that erects the phallus in ducks and roosters [8]. Reports on the corpus paracloacalis vascularis of the domestic fowl [12, 19, 20], turkey [11] and Guinea fowl [17] have been presented.

In the rooster, the paracloacal vascular body is a small, red oval structure located in the wall of urodeum close to the receptacle of the deferent duct; it is covered with a connective tissue capsule [12]. In light microscopy, the paracloacal vascular body reveals capsule, trabeculae, capillary cords and lymphatic spaces. The lymphatic spaces are divided into peripheral lymphatic spaces under the capsule and the internal lymphatic spaces in the parenchyma [19]. Lymphatic channels are present close to a group of capillaries [7]. The trabeculae have one or two arterioles and venules inside. SEM of corrosion casts reveal anastomosing vascular cords between which lay lymphatic spaces [19]. Transmission electron microscopy reveals capillaries and many pericytes in the cords with a layer of endothelial cells with rare fenestrations with diaphragm [19]. Gunawardana and Scott [7] also described fenestrations in the endothelial processes but in this instance they did not show any diaphragms. In the turkey, two units of paracloacal vascular body are located caudoventrally to the proctodeum [11]. This structure possesses large lymphatic sinuses. Several capillaries are inside the paracloacal vascular body and they are surrounded by lymph sinuses. In the turkey, the connective tissue cords also contain blood vessels and form a network that supports the intervening lymphatic spaces [11]. In the guinea fowl, the paracloacal vascular body comprises a capsule, peripheral and internal lymphatic sinuses, trabeculae and capillary cards. Arterioles and venules are present, and the capillaries display rare fenestrations [17]. For a review of the structure of the paracloacal vascular body see [8, 10].

The paracloacal vascular body is present in different avian species, the names given to it by different authors may differ. King [9] in Nomina Anatomica calls it "Corpus vasculare phalli", and refers to other names *"Glomera corporis vasculare phalli"*, *"Lymphobulbus phalli"*; this name was used also by Rautenfeld [15], *"Corpus paracloacalis vascularis"* [12, 19, 20], paracloacal vascular body [17]. In the ostrich, a structure equivalent to paracloacal vascular body has not yet been established. Reports from previous authors, did not elucidate on the issue [8].

The paracloacal vascular body received attention because of the absence of accessory sexual glands in male birds and the involvement of this body in the erection of the phallus [8, 20]. The paracloacal vascular body is believed to supply lymph to the phallus for erection in various avian species such as the fowl and the duck [8] and ostrich [15].

This study determined the distribution and structure of microvasculature in the phallus of the ostrich using India ink injection (Light Microscopy) and Transmission Electron Microscopy (TEM).

MATERIALS AND METHODS

The torsos of 18 sexually mature and active male ostriches, were obtained from the Oryx abattoir in Krugersdorp, Gauteng, in South Africa. The vascular system of the reproductive tract of six of the ostriches was flushed free of blood by injecting physiological saline through the descending aorta. The descending aorta was carefully perfused directly in its thoracic segment. The vessel was cannulated and physiological saline was injected into the arterial system.

For the study of the arterial system, the descending aorta of six birds were injected with India ink, and for the venous system, another six birds were injected with India ink through the caudal vena cava.

Tissue blocks of the *phallus* were prepared for light microscopy, selected areas were removed, trimmed into small blocks and immersion-fixed in 10 % buffered formalin for a minimum period of 48 hours. The tissue blocks were conventionally processed for light microscopy, and histological sections were stained with haematoxylin and eosin (H & E). The distribution of the arteries and veins was studied and described using an Olympus BH-2 microscope and appropriate information was digitally recorded with a Nikon Coolpix 4500 digital camera. The diameter of the vessels

was determined with the aid of a calibrated stage micrometer (Graticules Pyser-SGI Ltd., UK).

For transmission electron microscopy (TEM) the vascular system of the reproductive tract was flushed free of blood, in six birds, as described above. Small blocks of tissue were removed from the phallus and the sponge-like tissue (consisting of numerous lymph spaces traversed by connective tissue septa or struts), immersed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The specimens were stored at 4 °C prior to processing for TEM. In the lab the tissue blocks were conventionally processed for TEM.

RESULTS

General morphological features

The phallus, was attached to the ventral wall of the cloaca, it comprised of a base and a conical shaft. In general, the phallus comprises two fibroelastic bodies, one phallic sulcus, one elastic vascular body and one pair of muscles.

The phallus was lined externally by stratified squamous epithelium, and its root was surrounded ventro-laterally by a sponge-like tissue (Figs. 1, 2). The walls of the phallic sulcus were composed of erectile tissue, they were lined externally by stratified squamous epithelium (Fig. 3). The phallic sulcus was dorsally situated, it originated close to the papilla of deferent duct and extended until the tip of the organ.

Observations of blood vessels distribution

The naming of blood vessels (blood capillaries, arterioles, arteries, venules and collecting veins) was done according to the dimensional criteria supplied by Rhodini [16]. The root of the phallus was surrounded ventro-laterally by a sponge-like structure consisting of numerous large lymph spaces traversed by connective tissue septa or struts. The connective tissue struts were richly supplied with capillaries, relatively large arterial vessels (Fig. 1) as well as abundant venules and large collecting veins (Fig. 2).

The connective tissue septa were randomly arranged and varied in size and shape, but all were characterised by an extensive network of blood vessels as indicated above. The variably sized intervening lymphatic spaces communicated with each other forming an extensive lymphatic reservoir.

The walls of the phallic sulcus were composed of closely packed, regularly spaced mucosal folds covered by a stratified squamous epithelium. The surface epithelium was supported by a relatively wide layer of connective tissue characterised by an extensive network of small lymph spaces. This well-developed layer of erectile tissue displayed a zone of smaller blood vessels, mainly capillaries, lying beneath the epithelium, whereas larger, randomly distributed arterioles and a few sparsely distributed venules were scattered throughout (Fig. 3). When compared to the spongy tissue at the root of the phallus, the erectile tissue lining the phallic sulcus presented relatively few capillaries and venules. Numerous lymphocytes were also a feature of this tissue. The lymph spaces communicated with larger spaces situated in a deeper zone of connective tissue. This zone resembled some of the features of the spongy tissue described above, although the septa were more substantial, forming a greater proportion of the tissue. Numerous capillaries, arterioles and some larger arterial vessels were prominent within the septa. Prominent accumulations of colloid-like substance were obvious in the larger lymph spaces indicating the presence of lymph (Fig. 3).

The elastic vascular body located in the ventral part of the body of the phallus, exhibited a few arterial vessels (arterioles and capillaries) that were sparsely distributed in the elastic connective tissue. The elastic vascular body was continuous with the fibrous body which was composed of collagen fibres and poorly vascularised. However, it did demonstrate an erectile potential by virtue of a low incidence of lymphatic channels. The fibrous body did not exhibit blood vessels and contained collagen fibres, a few lymphatic channels (some of which contained lymph) and scant erectile tissue.

Ultrastructural features

The capillaries located in the connective tissue struts forming the spongy tissue at the base of the phallus (Figs. 4 B, C) displayed both wide and attenuated endothelial profiles. The most obvious morphological feature was the presence of numerous wide fenestrations beneath which the basal lamina remained continuous (Fig. 4 C). A diaphragm sealing the fenestrations could not be convincingly demonstrated in the material studied. Numerous cytoplasmic processes extended into the lumen from the surface of the endothelium. In certain areas the cytoplasmic processes appeared to enclose and sequester luminal contents. During this process the cytoplasmic extensions trapped material from the lumen and interiorized it into the endothelium,

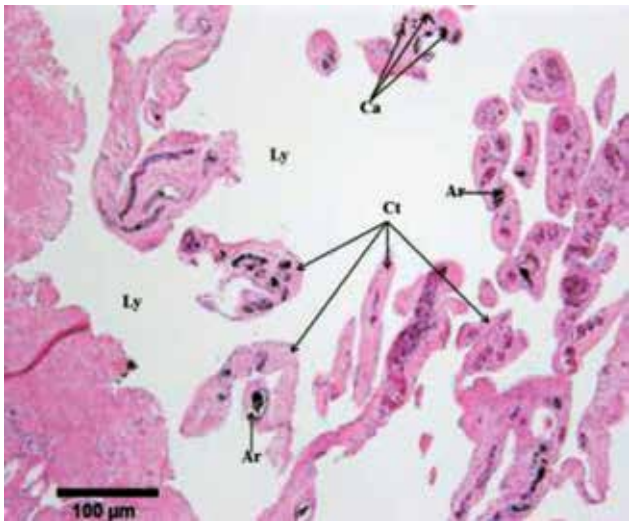

Fig. 1. Light micrograph of the spongy tissue at the root of the phallus. Note the variably sized connective tissue struts (Ct) with blood capillaries (Ca) and arterioles (Ar) filled with India ink. There are empty lymph spaces (Ly) between the struts

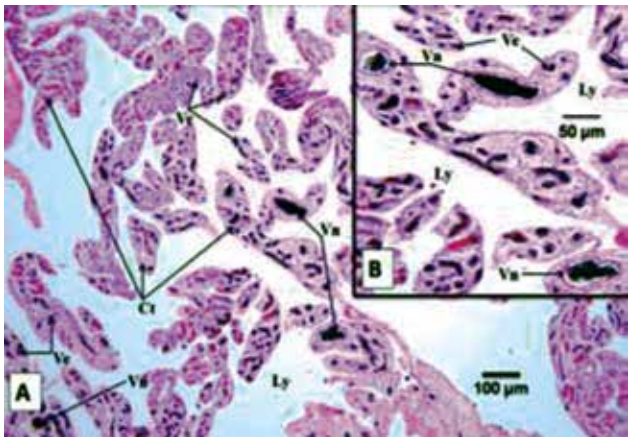

Fig. 2. A) Region similar to that shown in Fig. 5. but with India ink injected into the venous system. There are connective tissue struts (Ct) with abundant venules (Ve) and collecting veins (Vn) and the large intervening lymph spaces (Ly). B) Magnification of part of Fig. A) showing tissue struts with venules (Ve) and collecting veins (Vn). There are empty lymph spaces between the struts (Ly)

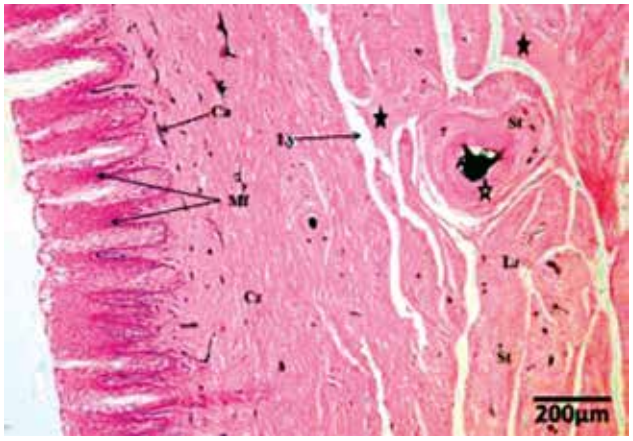

Fig. 3. Light micrograph of the phallic sulcus. There are regular mucosal folds (Mf) lining the surface, the more compact zone (Cz) with scattered capillaries (Ca) and the looser zone (Lz) with connective tissue struts (St) containing larger vessels (white star) as well as capillaries. Colloid-like substance (black stars) lies in the lymph spaces (Ly) between the struts. India ink injection.pinocytotic vesicles (stars), lumen (Lu), basal lamina (Bl)

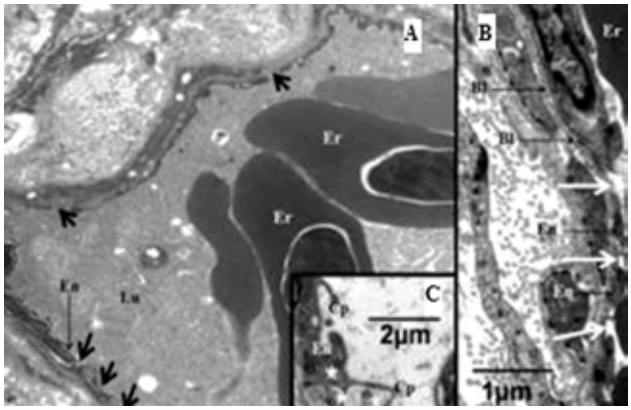

Fig.4. A) Venule in the spongy tissue at the root of the phallus displaying wide fenestrations (thick arrows) in the endothelium (En). Erythrocytes (Er) are packed in the vessel lumen (Lu). B) Longitudinal section of a capillary in the spongy tissue. The endothelium (En) is discontinued by wide fenestrations (white arrows) although the basal lamina (Bl) is continuous. C) A blood capillary displaying cytoplasmic processes (Cp) extending from the vessel wall (En) and which appear to be sequestrating luminal contents (stars)

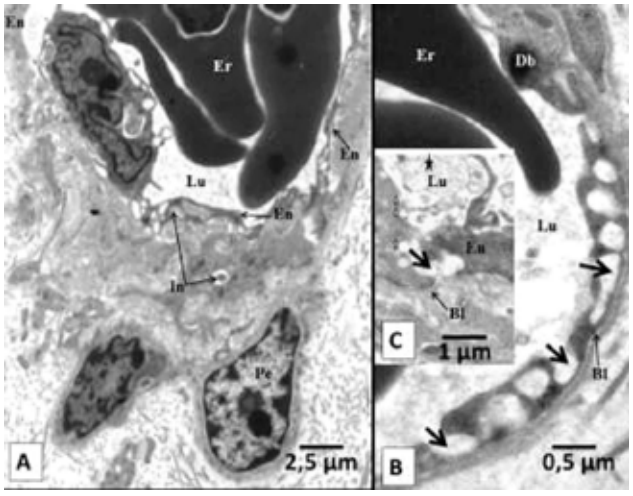

Fig. 5. A) Electron micrograph of a blood capillary in the erectile tissue of the phallic sulcus demonstrating complex interdigitations of the endothelium (En). Internalised material (In), lumen (Lu), erythrocytes (Er), presumptive pericyte (Pe). B) Higher magnification of part of the capillary wall in Fig. 2 A. There is apparent internalisation of luminal contents resulting in narrow strips of attenuated endothelium (short thick arrows) resting on a continuous basal lamina (Bl). Dense body (Db), lumen (Lu), erythrocytes (Er). Inset C) Higher magnification of the capillary endothelium (En) showing cytoplasmic processes in the act of internalising luminal material (black star). An endothelial fenestration (short thick arrow) is apparent as well as formation of pinocytotic vesicles (stars), lumen (Lu), basal lamina (Bl)

resulting in the formation of pale vesicle-like structures. The endothelium was extremely irregular in profile, and in some instances was composed of more than one layer of overlapping squamous cells joined by apical adhering junctions. In addition to the vesicles, the endothelium displayed mitochondria, lysosomes, profiles of RER and free ribosomes. Arterioles were structurally similar in appearance to the capillaries but also revealed dense bodies. Internalisation of luminal material was also observed.

Venules were generally lined by an attenuated endothelium which was broken in places by wide fenestrations (Fig. 4 A). A continuous basal lamina was again obvious. The endothelial cells displayed blunt luminal projections and regions of cellular overlap were marked by sparse adhering junctions. In addition to the standard cytoplasmic organelles, the cells also displayed occasional dense bodies as well as pinocytotic vesicles.

The vessels present in the erectile tissue lining the phallic sulcus exhibited features similar to those seen in the spongy tissue in respect of their basic structure and organelle content. However, the endothelial cells were markedly more intricate and irregular in design, displaying numerous interdigitating cytoplasmic processes, particularly in regions of overlapping cells. The capillary endothelium displayed numerous fenestrations as well as regions where apparent sequestration of luminal contents resulted in exceptionally attenuated stretches of endothelial cytoplasm. In both instances a well-developed, continuous basal lamina was apparent (Fig. 5 A, B, C).

DISCUSSION

A brief overview based on the available literature and on the histological observations from the present study is presented to provide proper perspective on the distribution of blood vessels in the phallus. This study provides the first information on the distribution and structure of the microvasculature of the ostrich phallus. The specialized morphology (gaps and fenestrations, irregular endothelium, internalization of luminal material) of vessels in the spongy and erectile tissue of the phallus are previously unreported features which appear to be consistent with the erection mechanism reported in the ostrich.

The spongy tissue that lies ventro-lateral to the root of the phallus in the ostrich is comparable to a concentration of tissue with similar structural features that has been referred to as the paracloacal vascular body in various avian species. This spongy tissue in the ostrich has been termed the "great erectile cushion" [8], *"lymphobulbus phallus"* [15] and *"lymphobulbus phalli"* in Nomina Anatomica Avium [9].

The paracloacal vascular body is considered to be an accessory organ of the phallus. It develops at puberty and transmits lymph from arterial vessels into the phallus, during tumescence [6]. Earlier authors suspected the presence of the paracloacal vascular body in the ostrich [8], but they failed to provide adequate evidence of its existence. This report therefore is the first to localize, describe the gross and histological features of this organ, as well as describe its microvasculature.

In the rooster, the paracloacal vascular body is a small, red, oval structure located in the wall of the urodeum, close to the receptacle of the deferent duct and is covered by a connective tissue capsule [12]. The sponge-like tissue was supplied by branches of the pudendal artery and drained by branches of the pudendal vein [3, 4].

When viewed by light microscopy, the paraclocal vascular body reveals a capsule, trabeculae, capillary cords and lymphatic spaces. The lymphatic spaces are divided into peripheral lymphatic spaces, under the capsule, and internal lymphatic spaces in the parenchyma [19]. The lymphatic channels or spaces are present close to groups of blood capillaries [7]. The trabeculae contain one or two arterioles and venules. These features were verified in the present study of the spongy tissue of the ostrich phallus, except that the connective tissue element was not obviously divided into capillary cords and trabeculae. In the ostrich, larger blood vessels were found together with capillaries. Scanning electron microscopy (SEM) of corrosion casts of the paracloacal vascular body confirmed the existence of anastomosing vascular cords, between which were lymphatic spaces [19].

The basic structure of the paracloacal body, namely, lymph-filled spaces separated by vascular-rich fibrous cords as described in the fowl [7, 19] has also been confirmed in the turkey [10] and in the guinea fowl [17]. Although Sugimura et al. [19] and Sasaki et al. [17] observed only a few fenestrations in the blood capillary endothelium of the fowl and guinea fowl respectively, Gunawardana and Scott [7] described numerous fenestrations without closing membranes in the rooster. A similar situation was observed in the ostrich therefore supporting a previously held view [11] that fluid leaves the

capillaries by diffusion rather than by active transport. It should be conceded, however, that the large numbers of pinocytotic vesicles noted in the ostrich blood vessels certainly suggests that active transport of fluid may play a supplementary role.

The specific morphological features of the spongy tissue described in this study, as well as the particular distribution of abundant, variably-sized capillaries and venules with fenestrations, would suggest that this structure participates in the mechanism of erection of the phallus in the ostrich, as has been demonstrated in other avian species [6, 7, 12, 13, 17, 20]. That the spongy tissue at the root of the ostrich phallus supplies the fluids for erection is based on the presence within the spongy tissue (lymphobulbus phalli) of lymphatic vessels (lymph channels), as well as connective tissue strands with arterial vessels (capillaries and arterioles) and venous vessels (venules and collecting veins) that are richly supplied with fenestrations.

This study therefore confirms the existence of a paravascular cloacal body in the ostrich, and also that the mechanism of erection of the phallus in the ostrich is lymphatic as previously reported by Rautenfeld [15]. The use of the term *"lymphobulbus phalli"* in respect of the ostrich phallus [9] is thus supported.

The erectile tissue in the sulcus of the phallus exhibits the same morphological features as that of the *lymphobulbus phalli*, although in the latter, this tissue is more abundant, more complex, and is the main source of lymph for phallic erection in the ostrich. Additionally, there was no indication that the erectile tissue in the *sulcus phalli* communicates with the elastic vascular body that lies ventrocaudal to the shaft of the phallus. In the present study, the elastic vascular body did not reveal much erectile tissue, in contrast to earlier observations that the elastic vascular body contains an inner core of erectile tissue [8].

CONCLUSIONS

In conclusion, this study demonstrated that the *lymphobulbus phalli* and erectile tissue of the phallic sulcus, the presence of many fenestrations in the capillaries, arterioles and venules reflect the potential for rapid fluid exchange consistent with the process of erection and detumescence. Additionally, the relatively loose arrangement of overlapping endothelial cells connected by only a few adhering junctions in these vessels would also assist in the rapid drainage of lymph from the lymph spaces to the blood circulation during detumescence.

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TOTAL ANTIBIOTICS — A NEW POSSIBLE ALTERNATIVE FOR THE SCREENING OF COCCIDIOSTAT RESIDUES IN POULTRY MEAT

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ABSTRACT

The Total Antibiotics test is a microbial inhibition test which has been recently introduced for the detection of antibiotics in meat. The aim of this study was to determine whether it would be suitable for the detection of coccidiostats in poultry meat. A comparison with the Premi®Test was assessed also for the suitability of the detection of coccidiostats in poultry meat. A selection of poultry meat samples of different organ parts were assessed with 14 samples from Slovakian farms that had previously been tested for coccidiostats by the Veterinary and Food Institute in Košice. In addition, another 8 samples from varied Slovakian supermarkets such as Lidl, Billa and Tesco with samples of chicken or duck meat, were tested. Each prepared sample was added to the Total Antibiotics kit tubes and incubated. The samples from all sources showed a mixture of positive and negative results for the detection of coccidiostats.

For the Premi®Test, the samples used the same extraction procedure as the Total Antibiotics, placed in Premi®Test kit tubes and incubated. The Premi®Test demonstrated a mixture of positive and negative results, as similar to the Total Antibiotics for coccidiostats in the poultry farm samples. However, the Premi Test revealed many more negative results for the supermarket sources compared to the Total Antibiotics. Therefore, based on the total number of positive results, we concluded that Total Antibiotics is more sensitive for the detection of coccidiostats in poultry meat, but depending on the source of the samples, both Total Antibiotics and Premi®Test had either similar or opposite results for the detection of coccidiostats.

Key words: coccidiostats; detection; poultry; residues

INTRODUCTION

The human consumption of poultry meat has increased over the years due to an increased population and demands for poultry meat. Also, the availability of poultry meat has become more available for consumers to purchase. However, the requirements for large production of poultry

within a short time frame cannot, sometimes, meet the demand. This is because poultry flocks have to meet welfare and hygienic demands so they can be stated fit to eat. Many veterinary drugs and prophylactic treatments are given to prevent the spread of poultry diseases [6].

Coccidiosis is a serious disease of poultry that can cause huge economic losses particularly where birds are reared intensively. To avoid the disease, coccidiostats are routinely given in poultry feed prophylactically and there is concern that this may lead to residues in poultry meat and eggs and present a risk for consumers. The farmer does prevent the parasitic disease with preventive measures of administrating coccidiostats into routine care, however the withdrawal period of coccidiostats before slaughter needs to be determined to prevent the accumulation of coccidiostat residues [5].

The European Union (EU) has many regulations governing food and feed safety. Regulation (EC) No. 1831/2003 [8] is regarding additives for use in animal nutrition; only additives that have been through a specific authorisation procedure may be placed on the market. The Regulation (EC) No. 1831/2003 followed food and feed safety requirements outlined in Regulation (EC) No. 178/2002 [7] by validating a 10 year authorisations of coccidiostats which are included in the additives regulated by this legislation. The regulation also set up EU Reference Labs for the testing of feed additives. Currently the EU has granted 28 authorisations for 11 coccidiostats for different species and uses. The authorisation contains information such as: the characteristic of the coccidiostat, maximum, minimum and recommended dosages, labelling, withdrawal periods and maximum residue limits (MRLs) if required [2, 9].The safety of the use of coccidiostats has been extensively assessed by the European Food Safety Authority (EFSA).

EU member states are legally obliged to control the residues of veterinary medicinal products including coccidiostats in food and feed under the Council Directive 96/23/EC [3]. As with the screening of food of animal origin for human consumption, it is important that the methods used for the screening must be: rapid, allow a high throughput of samples, and relatively simple sample preparations. The current residue control strategy is based on an initial qualitative screening test followed by a quantitative confirmatory test [1].

Microbial inhibition tests (MITs) are used as part of the qualitative screening and they are based on the principle that the growth of a strain of bacteria present in the agar can be inhibited by the presence of an antimicrobial (inhibitory) substance. These tests can be plate tests or tube tests. The tube tests are very popular commercially and produce broad spectrum screening tests. They are designed to detect most of the relevant antimicrobial compounds, such as antibiotics or chemotherapeutics in food of animal origin at or below their MRLs within 3–4 hours. A positive result is presented by the absence of colour change from purple to yellow of the indicator (bromocresol purple) in the agar medium. The major bacterial strain used in the tube tests is Bacillus stearothermophilus var. calidolactis.

Premi®Test and Total Antibiotics are examples of this type of tests. The Premi®Test was developed by DSM Food Specialties (Delft, the Netherlands) and it is by far the most widely used test for the rapid screening of antibiotic residues in meat samples. The Total Antibiotics is one of the newest tube tests on the market developed by the Eurо-Clone S. p. A. (Pero, MI, Italy) for the same purpose. Both tests use Bacillus stearothermophilus var. calidolactis as the bacterial strain. In order to gain more insight in the detection capacity of the test Total Antibiotics, the aim of our study was to evaluate this test for the possible screening of coccidiostat residues in poultry meat. The Premi®Test was also assessed and compared with the Total Antibiotics for its suitability in the detection of coccidiostat residues in poultry meat.

MATERIALS AND METHODS

Poultry meat samples

To determine whether the Total Antibiotics and the Premi®Test would be suitable for the detection of coccidiostats in poultry meat, a selection of poultry meat samples from Slovakia was made. A total of 22 samples of poultry tissue were collected during the time period from November 2015 to February 2016 as follows: 14 samples were from Slovakian farms that had previously been tested for coccidiostats by the Veterinary and Food Institute in Košice, 4 samples were from Lidl, 3 samples were from Billa and 1 sample was from Tesco. For the control, a negative breast chicken sample was used. A variety of poultry tissues [chicken breast (6), duck fat and skin (1), duck breast (1), chicken thigh (4), chicken fat and skin (2), chicken spleen (2), chicken gizzard (2), chicken kidney (1), chicken liver (2), and chicken heart (1)] were stored in a freezer at –20 °C until the analysis.

Preparation of samples

All of the samples for both MIT tests used the same preparation method as for the Total Antibiotics. Poultry meat samples were thawed and 2.5 g of meat was weighed in a tube and minced with a sterile lancet. The extraction buffer provided from Total Antibiotics was diluted 1 : 10 with demineralised water. Ten ml of freshly prepared extraction buffer was poured into the test tube with the minced poultry tissue and foil tops were placed on top. The test tubes were vortexed for half a minute and incubated for 2 hours at 37 °C. The clean supernatant was collected and used in the respective tests.

Total Antibiotics

Product detail: A commercial antibiotic broad spectrum test kit supplied by EuroClone S. p. A. (Italy) containing 96 ready-to-use capped test tubes with Bacillus stearothermophilus var. calidolactis in a solid agar medium and an extraction buffer concentrate were used for the preparation of the samples for the residue analysis. The Total Antibiotics test tubes and extraction buffer must be stored in the fridge at 4 °C before use.

Test procedure: 200 µl of clear supernatant taken from the surface of the previously prepared sample was pipetted with a single disposable pipette to each of the Total Antibiotic kit test tubes. The test tubes were tightly closed with a yellow cap and incubated in a pre-heated thermostatic dry bath at 65 °C for 3 hours. The sample incubation time can be extended until the negative control has changed colour.

The interpretation of results: Results were read by analysing the agar medium present in the Total Antibiotic test tubes. The incubation period has to be completed and stopped if the agar medium in the negative control has changed to a clear yellow colour. If the colour of the agar medium remains unchanged (purple), the sample is labelled positive. All shades of purple are also read as positive samples. Complete change in medium colour from purple to yellow is labelled negative. Photos of samples can be made directly after reading the samples to ensure that no changes had taken place.

Premi®Test

Product detail: A commercial antibiotic broad spectrum screening test kit supplied by R-Biopharm AG (Germany) containing 25 ready-to-use ampoules with Bacillus stearothermophilus var. calidolactis in a solid agar medium was also used to test the same samples.

Test procedure: 100 µl of clear supernatant taken from the surface of the previously prepared sample was pipetted with a 100 µl pipette to each of the Premi®Test kit test tubes. The test tubes were covered with sealing plastic foil and incubated in pre-heated thermostatic dry bath at 65 °C for 3 hours. The sample incubation time can be extended until the negative control has changed colour.

Interpretation of results: Results are read by analysing the agar medium present in the Premi®Test test tubes. The incubation period has to be completed and stopped if the agar medium in the negative control has changed to a clear yellow colour. If the colour of the agar medium remains unchanged (purple), the sample is labelled positive. All shades of purple with no clear colour change are considered dubious. All dubious results are also suspected samples but the concentration of coccidiostats or antibiotics is at the level of detection limit of the test (not so high but still detectable according to the presence of the violet colour). If the colour of the agar medium has a clear change to the yellow colour, the sample is consider negative. Photos of samples can be made directly after reading the sample to ensure no changes take place.

RESULTS

The first set of results was done with samples from the Slovakian farms. The results of the screening for the presence of coccidiostat residues in poultry meat by MIT Total Antibiotics and Premi®Test are presented in Table 1.

The samples showed that both the Total Antibiotics and the Premi®Test had both negative and positive results. All two MIT kits were functional in detecting the presence of coccidiostats in the chicken tissues. Samples 1, 2, 9, and 10 representing the breast muscle and thigh muscle were negative for the presence of coccidiostats by both MITs. Samples 3, 4, 5, 11, 12 and 14 representing the chicken fat and skin, spleen and gizzard were positive for the presence of coccidiostats by both MITs. Sample 7 and 13 representing the chicken liver were positive for the Total Antibiotics and dubious for the Premi®Test. Sample 6 representing chicken kidneys was negative for the Total Antibiotics and dubious for the Premi®Test. Sample 8 representing the chicken heart was positive for the Total Antibiotics and negative for the Premi®Test. All samples with dubious results were suspected samples for the presence of coccidiostat residues.

Table 1. Results of screening for coccidiostats with MIT of first batch of samples

	Sample (number/matrix)	Total Antibiotics	Premi®Test
1	CHBM	–	–
2	CHTM	–	–
3	CHF&S	+	+
4	CHS	+	+
5	CHG	+	+
6	CHK	–	±
7	CHL	+	±
8	CHH	+	–
9	CHBM	–	–
10	CHTM	–	–
11	CHF&S	+	+
12	CHG	+	+
13	CHL	+	±
14	CHS	+	+

+ — positive sample; ± — dubious sample; – — negative sample
CHBM — chicken breast muscle; CHTM — chicken thigh muscle; CHF&S — chicken fat and skin; CHS — chicken spleen; CHG — chicken gizzard; CHK — chicken kidney; CHL — chicken liver; CHH — chicken heart

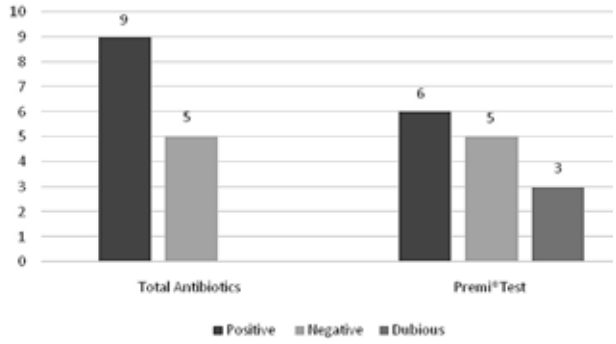

Fig. 1. Bar chart showing the differences of positive, negative and dubious results from the first batch of samples

Table 2. Results of screening for coccidiostats with MIT of second batch of samples

	Sample (number/matrix)	Total Antibiotics	Premi®Test
1	CHTM	–	–
2	CHTM	+	–
3	CHBM	–	–
4	CHBM	–	–
5	CHBM	+	–
6	CHBM	+	–
7	DBM	+	–
8	DF&S	+	+

+ — positive sample; – — negative sample
CHBM — chicken breast muscle; CHTM — chicken thigh muscle; DBM — duck breast muscle; DF&S — duck fat and skin

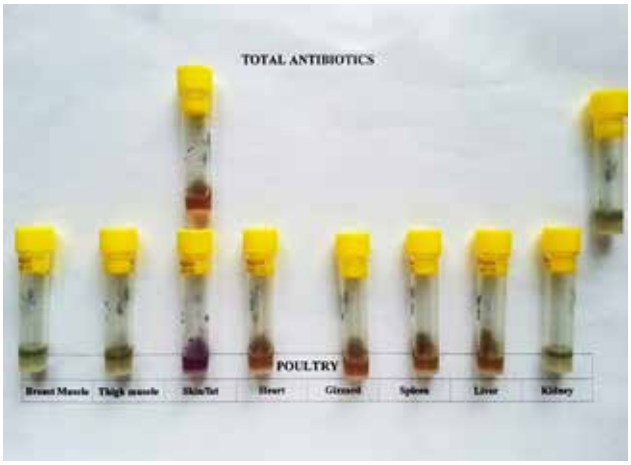

Fig. 2. Results from first batch of samples testing with Total Antibiotics

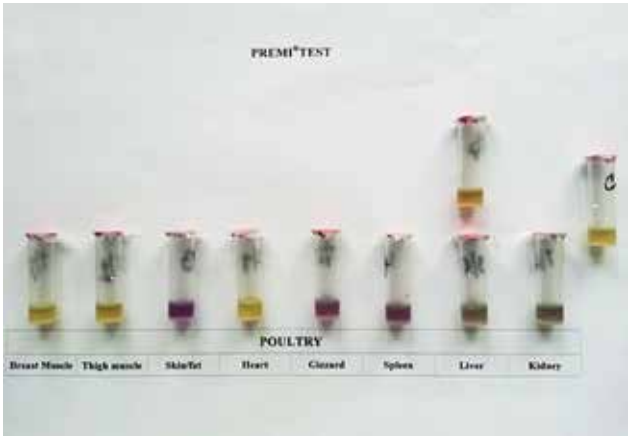

Fig. 3. Results from first batch of samples testing with Premi®Test

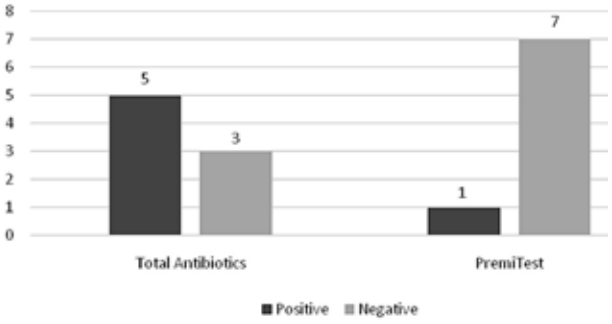

Fig. 4. Bar chart showing the differences of positive and negative results from second batch of samples

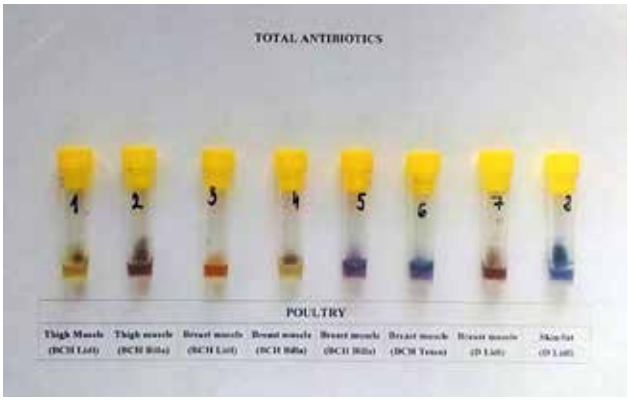

Fig. 5. Results from second batch of samples testing with Total Antibiotics

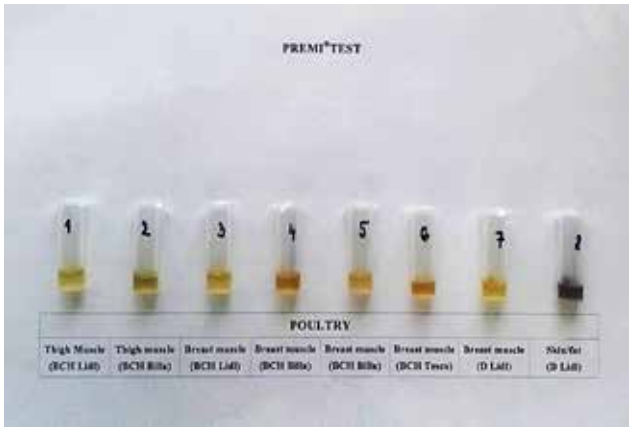

Fig. 6. Results from second batch of samples testing with Premi®Test

Figure 2 shows the resulting colour change in the agar medium for the Total Antibiotics. Figure 3 shows the resulting colour change to the agar medium for the Premi®Test.

The bar chart below in Figure 1 shows the differences of positive, negative and dubious results shown in Table 1.

The second set of results was done with samples from different Slovakian supermarkets (Lidl, Billa, Tesco). The results of the screening for the presence of coccidiostat residues in poultry meat by MIT Total Antibiotics and Premi®Test are presented in Table 2.

Figure 5 shows the resulting colour change in the agar medium for the Total Antibiotics. Figure 6 shows the resulting colour change to the agar medium for the Premi®Test.

The samples also showed that both the Total Antibiotics and the Premi®Test had both negative and positive results. All two MIT kits were functional in detecting the presence of coccidiostats in the chicken and duck tissues. Samples 1, 3, and 4 representing the chicken thigh muscle and breast muscle were negative for the presence of coccidiostats by both MITs. Sample 8, representing the duck fat and skin, was positive for the presence of coccidiostats by both MITs. The other four samples (2, 5, 6, 7) representing the chicken thigh and breast muscles and duck breast muscle were positive only for the Total Antibiotics. No dubious results were detected after screening.

The bar chart below in Figure 4 shows the differences of positive, negative and dubious results shown in Table 2.

DISCUSSION

In this study of establishing the performance of the newly developed MIT, the Total Antibiotics and a comparison with the commonly used MIT, Premi®Test was made. The comparison was done to evaluate the efficiency and sensitivity of the Total Antibiotics by using the same samples of poultry meat.

The sample preparation for both MIT was the same. The extraction buffer from the Total Antibiotics kit was used to extract the contents of the tissue and convert it into a clear supernatant solution so it could be used in both MIT. The extraction buffer must be correctly diluted with demineralized water (9 parts water and 1 part extraction buffer). If normal tap water or bottled water was used, this could contaminate the extraction buffer and serve as a "carry over" with the samples once mixed. Samples must be minced into smaller pieces so the surface area of the poultry meat is high. Ampoules/test tubes used in the kit must be handled with care and stored in the fridge at 4 °C and if the medium

agar had changed colour inside the well before use then the ampoule must be discarded. To ensure the extraction buffer and the ampoules used for both the Premi®Test and the Total Antibiotics were suitable to be used in residue detection, the kits must be kept at 4–6 °C. Manipulation of the kits were handled with care.

Each test run in the study had one negative control. The negative control was the same sample in all series of screening. This was done to ensure fair and consistent results and recognition once the colour had changed. The negative control was a breast muscle sample from a chicken confirmed free of bacteria at the Veterinary and Food Institute in Kosice. The first incubation at 37 °C of the extraction of poultry meat was put into an incubator which was stable at this set temperature. The incubator had a sealed door to ensure no escape of heat could exit and no external air draughts could enter. In addition, no preheat treatment was required with the incubator. During the second incubation at 65 °C, only 2 °C below and above was allowed to change in the thermostatic dry bath. All series of MITs performed were kept within the margins of 65 °C and therefore were constant because the thermostatic dry bath was electronically automated. Before each run of the test tubes, the thermostatic dry bath was preheated before the three hour time set. If the negative control had not changed to a clear shade of yellow then the incubation time was prolonged. In this study, the prolongation time extended for another 30 minutes for the second batch of samples. Ensuring stable temperatures were run in accordance to the kit manual so the efficiency of the MIT could be analyzed successfully and recognized as a good performance of the assay.

To avoid cross contamination of meat samples and the "carry over" of substances used to prepare samples, gloves were worn. The lancet was cleaned and disinfected with ethanol after mincing each sample. In addition, the cutting board was wiped clean in between sample preparation with ethanol.

Deposition of the supernatant was handled with care. The micropipette used was automatically set to the exact amount of supernatant needed to be collected and a disposable pipette was used in each one. This was to ensure the correct amount and prevent cross contamination of the supernatant. The supernatant was pipetted onto the top of the medium without touching the agar. Yellow tops for the Total Antibiotics were closed properly and for the Premi®Test sealing tape were applied to tightly seal the tubes. This was done to prevent any evaporation during the second incubation period.

The Total Antibiotics overall was found to be a more sensitive tests with both batches of the samples. Making comparisons with the more commonly used MIT, Premi®Test was a good indication to use as a comparison for the suitability of the Total Antibiotics.

With the first batch of samples, more than half of the samples were found to be positive in the Total Antibiotics in comparison with the Premi®Test. With the second batch of samples, more than three quarters of the samples were positive in the Total Antibiotics in comparison with the Premi®Test. The Premi®Test had most of the samples as negative, whereas the Total Antibiotics had more positive samples. This showed that the Total Antibiotics was able to detect the residues much more efficiently in comparison to the Premi®Test.

The Total Antibiotics is rapid, simple and multi-residually. The kit is suitable to screen multiple samples, and able to complete an assay run within a few hours. If consistent reading of the results was made, the Total Antibiotics overall showed a better detection to pick up the concentration of coccidiostat residues in all types of poultry tissues including different species. According to Gondova et al. [4], the Total Antibiotics has an increased sensitivity compared to other tube tests because it corresponds with the MRLs (however a confirmatory step afterwards is still essential) and there are a reduced number of false positive results as the sample preparation is simple and the extraction buffer is efficient in extracting the residue from the meat sample. With the positive samples used in our study, the second confirmatory test could be made so concentrations and individual identification of coccidiostats could be done.

CONCLUSIONS

The production of poultry meat is carried out to meet the consumer's demand for their nutritional health. The effects of coccidiosis on the poultry industry has detrimental effects on the number of poultry and the cost of rearing. The production of coccidiostats is widely used to control the spread of the protozoan parasites among the poultry flocks. However, the accumulation of coccidiostats in poultry is also toxic and if passed along the food chain for human consumption, then this can have negative impact in the quality of meat and increase health risks for consumers,

especially for those on a diet mainly of poultry meat. It is up to the Regulations set by the EU to make standards so they can be referred to during the manufacturing process.

In this study, the analysis of the presence of coccidiostats was determined because of the impact of the "carry over" of veterinary drug residues and specifically coccidiostat residues in poultry meat. The analysis of premixes, feeds and poultry meat can be done to determine the presence of coccidiostats and their residues in every sample to be tested. The analysis of the Total Antibiotics was made as it is a new and recently developed commercial MIT kit. Comparisons to the most commonly used MIT kit (Premi®Test) was made as it utilizes tube tests similar to the Total Antibiotics.

The presence or absence of coccidiostat residues in each sample involved a qualitative colour change of the agar medium. Making the analysis of the colour changes could be slightly different between different observers. Therefore, a colour scheme was devised. The results were recorded as either negatives or positive with the Total Antibiotics and positive, dubious or negative with the Premi®Test.

Poultry samples from the Slovakian farms (1st batch) and from the Slovakian supermarkets (2nd batch) provided a diverse source of samples to test. Poultry from Slovakian farms were tested by a confirmatory analysis before and many were found to be positive. However, poultry from Slovakian supermarkets should all be negative according to the EU Regulations because it is sold to the consumers.

Unfortunately, the Total Antibiotics was only able to detect the presence of a coccidiostat or of multiple coccidiostats in the sample; it was unable to determine the level of coccidiostats or if multiple coccidiostats were present in the sample. It is a rapid screening procedure which could be used, as it can still abide by the Regulations set by the EU. The test has the ability to detect the substances at their MRLs, however the confirmatory test afterwards need to be done by another laboratory test.

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OCCURRENCE OF ENDOPARASITES IN INDIGENOUS ZAMBIAN DOGS

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ABSTRACT

This study was conducted in the country of Zambia, Southern Africa, to investigate the occurrence of endoparasites in indigenous Zambian dogs. Faecal samples were collected from 41 indigenous Zambian dogs from different areas of the Mbabala region in the Southern province of Zambia during the "hot wet" season, although at the time that the samples were collected, the country was experiencing a drought. Faecal samples were analysed using the concentration flotation method with zinc sulphate for the determination of the presence of gastrointestinal parasites. The most prevalent parasites were species from the family Ancylostomatidae (65.0 % infection rate) which followed by: *Isospora canis* (9.8 %), *Dipylidium caninum* (4.8 %), and *Toxascaris leonina* (2.4 %). There were in addition, two cases of co-infections with the family Ancylostomatidae and *D. caninum*, as well as the family Ancylostomatidae and *I. canis*.

Key words: dog; endoparasite; family Ancylostomatidae; *Toxascaris leonina*; *Dipylidium caninum*; *Isospora canis*

INTRODUCTION

Parasitism is the most prevalent canine disease worldwide, negatively impacting not only its canine host [11] but also humans due to their potential to act as a zoonotic reservoir [3, 7]. In the large rural areas of Zambia, it is common to find a large population of malnourished free-ranging dogs. This situation combined with a general lack of animal welfare education, poor veterinary services and widespread poverty in the rural regions, exacerbates this health risk significantly [7].

The aim of this study was to investigate the occurrence of endoparasites in indigenous Zambian dogs. This constitutes the first study of its kind being conducted in this region.

MATERIALS AND METHODS

This study was conducted in Zambia, a Southern Africa country located between 22° and 34° East and 8° and 18° South (Fig. 1). Specifically, it took place in the Southern

Fig. 1. Zambia, geographical location of the sampling region is indicated by the star [2]

Fig. 2. A gash from an encounter with a baboon
(Original photo)

province of Zambia in the rural villages near the Mbabala town of Choma district [2].

Zambia has a subtropical climate with 3 distinct seasons: The "cool dry" season which is the winter equivalent (between May and July) with mean daily temperatures falling between 15 and 27 °C, followed by the "hot dry" season (from August to October) with temperatures peaking at 27—32 °C [5].

Samples were obtained and analysed during the "hot wet" season (known as "the rainy season") as 95 % of the annual precipitation occurs during these months. It runs from November to April (803 mm of rain on average yearly) [9], however at the time of our sampling, the country was experiencing a drought.

Individuals that owned one or two dogs used in this study tended to be keeping them for companionship and personal security. However, there were two cases where larger packs of up to 6 dogs were owned to protect crops from animals such as baboons and for hunting — both legal and illegal (Fig. 2). There were also a few statements made that the dogs were occasionally a source of income by selling them to the Chinese quarry team located nearby. The dogs were all kept outside and very rarely allowed in the house beyond the veranda. Very few had visited the local veterinarian; for those that had received veterinary attention it was for rabies vaccination. No dogs had been given therapeutic or prophylactic worming medication due to a lack of education, widespread poverty and their isolated location. Upon receiving the information from the owner that the dogs had never been treated for endoparasites they were given free deworming medication as a reward for their cooperation with this study. On the whole, all dogs surveyed were in poor condition, malnourished with evidence of ectoparasites (fleas and ticks), scars and occasionally obvious sores that warranted immediate medical attention. They were not receiving any veterinary assistance due to their rural location and the veterinary bill being beyond the reach of their income.

All samples were collected and analysed in the Southern province of Zambia from 28 different owners in 3 separate villages. Faecal samples were all taken *per rectum* with a lubricated gloved hand. The faeces were contained in a double glove and immediately placed in a cool box with ice packs. Each sample was assigned a number and further details recorded such as name of the dog and owner, as well as their specific location, size of the dog, breed, sex, age, reason for ownership, body condition and veterinary treatment history. Later, the samples were transferred into a third plastic bag and kept in the refrigerator at 4 °C for a maximum of 5 days.

The samples were later subjected to the concentration flotation method with zinc sulphate and analysed under a microscope where eggs/oocysts were identified and the intensity of the infection was gauged as follows: low (one egg per drop), medium (10 eggs per drop), high (5 eggs per 20× magnification field) [6].

RESULTS

The parasites found in the coprological examination included helminth eggs of the family Ancylostomatidae, *Dipylidium caninum*, and *Toxascaris leonina*, as well as oo-

Table 1. Adult dogs with overall species prevalence and prevalence per sex

Species	Prevalence – total [%]	Male [%]	Female [%]
Family Ancylostomatidae	21 (67.7)	10 (71.4)	11 (64.7)
Isospora canis	3 (9.7)	2 (14.3)	1 (5.9)
Toxascaris leonina	1 (3.2)	1 (7.1)	0 (0)
Dipylidium caninum	2 (6.5)	2 (14.3)	0 (0)
Negative	7 (22.6)	1 (7.1)	6 (35.3)
Total	**31**	**14**	**17**

Table 2. Young dogs with overall species prevalence and prevalence per sex

Species	Prevalence - total [%]	Male [%]	Female [%]
Family Ancylostomatidae	7 (70)	2 (66.7)	5 (71.4)
Isospora canis	1 (10)	1 (33.3)	0 (0)
Toxascaris leonina	0 (0)	0 (0)	0 (0)
Dipylidium caninum	0 (0)	0 (0)	0 (0)
Negative	2 (20)	0 (0)	2 (28.6)
Total	**10**	**3**	**7**

Table 3. Total prevalence of endoparasites in dogs

Species	Total prevalence [%]
Negative	9 (22.0)
Family Ancylostomatidae	27 (65.9)
Dipylidium caninum	2 (4.9)
Isospora canis	4 (9.8)
Toxascaris leonina	1 (2.4)
Mixed infections	
Ancylostomatidae + *Dipylidium caninum*	1 (2.4)
Ancylostomatidae + *Isospora canis*	2 (4.9)

cysts of *Isospora canis*. By far the most prevalent parasite were species from the family Ancylostomatidae, where the highest prevalence (67.7 %) were noted. Table 1 shows the prevalence of the parasite species in the 31 adult samples examined. *Isospora canis* had an overall prevalence of 9.7 %. *Toxascaris leonina* and *Dipylidium caninum* were not found in the adult females; however in the adult males. *D. caninum* was found at a 14.3 % prevalence and *Toxascaris leonina* at a 7.1 % prevalence. Overall 22.6 % of the dogs were negative with many more females (6 cases) being negative than the males (1 case).

The young also displayed a population sample largely infected with the family Ancylostomatidae (70 %) (Table 2). More females (7) were found for sampling than males (3) with the overall sample size of 10 being lower than that of the adults which had 31 samples. There were no positive cases for *T. leonina* or *D. caninum* with just one young male being found to be infected with *Isospora canis*.

Table 3 below shows that there was a 78 % chance of a dog from our sampling region testing positive for a gastrointestinal parasite. Within these positive dogs there was a 66 % chance that the parasite belonged to the family Ancylostomatidae. Furthermore, species from the family Ancylostomatidae were involved in all the co-infections with *D. caninum* and *I. canis*. *D. caninum*, *I. canis* and *T. leonina* had a fairly low prevalence, with *I. canis* being the next most common after the family Ancylostomatidae, followed by *D. caninum* and *T. leonina*.

There were no co-infections found in the young dogs. There were three cases of co-infections which we found in 2 males and in 1 female.

DISCUSSION

Nonaka et al. conducted a similar study in other regions of Zambia [7] and also found that the eggs of the Ancylostomatidae family were by far the most abundant in their study with 43.3 % on average. Our study region showed an even higher prevalence of 66 %. As only the classical faecal egg examination was undertaken in this study, it was not possible to distinguish between the morphologically similar eggs of the Ancylostomatidae family. A second study conducted by Islam and Chizyuka [4] further North in Zambia found the species to be *Ancylostoma caninum* and *Ancylostoma braziliense*. They found a much lower prevalence of *Ancylostoma* spp. eggs (10 %) and other helminths; however, they acknowledged that there selection of dogs were those from a better cared for and more restricted background than those used in other studies [4].

All of the parasite species detected in our study were also found in other regions of Zambia by other authors, however, their studies exhibited a larger species richness [4, 7]. They detected the following additional parasites: family Teniidae, *Toxocara canis*, *Diphyllobothrium* spp., *Spirocera lupi*, *Echinococcus granulosus*, *Sarcocystis* spp., *Physaloptera* spp., *Capillaria* spp., *Mesocestoides* spp., *Ascaris* spp., *Trichuris vulpis*, and *Schistosoma mansoni*. The increased richness may be as a result of their more advance detection techniques including coproantigen ELISA for Echinococcus and PCR, as well as a much larger sample size of 540 dogs.

The high prevalence of the family Ancylostomatidae is of particularly significant due to its zoonotic potential. Man is an accidental host and larvae cannot reach full maturity within them; however *Ancylostoma caninum* can cause eosinophilic enteritis and *A. braziliense* which is the primary cause of cutaneous larva migrans [9]. The possible reasons for its high prevalence may be due to the multiple modes of transmission including direct ingestion, per cutaneous, via paratenic hosts such as rodents and in the case of *A. caninum*, the additional possibility of transmammary, as well as lactogenic transmission [8]. Transmammary and lactogenic transmission may also help explain its almost equally high prevalence in the young (70 %) as well as the adults (67.7 %). Species of the family Ancylostomatidae are prolific egg layers with the potential to pass millions of eggs into the environment daily [9] and as all dogs in this study freely roam over large territories, there is a very high chance of them obtaining an infection.

Infectious concentrations of the family Ancylostomatidae eggs were higher in the adults with 60 % of adults having a medium concentration of infection in the samples which was 30 % higher than that of the young dogs. Furthermore, 25 % of the adults were found to have a high intensity of infection, whereas only 10 % of the young dogs had similar results. This may be as a consequence of the dogs having an increased chance of exposure of obtaining an infection orally or per-cutaneously over time.

When comparing adult males and females for the family Ancylostomatidae, the two tailed P-value of 0.369 indicates that there is no significant difference between the sexes. Statistical analysis in the other categories were not calculated as the expected values from the null hypothesis fall below 5 making it an inadequate statistical test.

Dipylidium caninum was not detected in the young but it was found in 4.9 % of the adults, Islam and Chizyuka [4] found a much higher prevalence of 25 %, however Nonaka et al. [7] found a more similar prevalence (2.2 %) to our results. It seems surprising that the infection rate was not higher considering that dogs had never been given anti-ectoparasitic treatment and most of them were visibly infected with fleas, which are intermediate host of *D. caninum* [1]. *D. caninum*, *I. canis* and *T. leonina* are not transmitted lactogenically or prenatally [9, 10], which would contribute to their lower prevalence in younger dogs.

CONCLUSIONS

It was found that the Mbabala region in the South-West of Zambia was no exception to the large prevalence of gastrointestinal endoparasites in tropical and subtropical regions of the world [10]. Eggs of the family Ancylostomatidae were largely prevalent with the young and adult rural dogs standing a very high chance of infection. The chance of getting infected with *Isospora canis*, *Dipyllidium caninum*, and *Toxascaris leonina* infections were much lower with adults having a higher risk of obtaining an infection than the young. Co-infections were not common but were detected in 3 adult dogs.

Education on the zoonotic risks and the need for anti-helminthic drugs was minimal and therefore needs to be encouraged. However, due to the fact that the vast majority of homes in these rural areas live in abject poverty, the drugs required are completely unaffordable to the owners, thus emphasizing the need to develop a program of free treatments. It is not so much a question of willingness but rather affordability.

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INFLUENCE OF PERORAL SUPPLEMENTATION OF SELENIUM AND VITAMIN E ON THE ANTIOXIDANT STATUS OF RACING PIGEONS

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ABSTRACT

The racing season is considered a critical period for racing pigeons due to the susceptibility to stress and weakening of the birds. One of the ways how to support their health and avoid problems involves supplementation of mineral-vitamin preparations based on selenium and vitamin E, which act as important antioxidants and protect cells against damage. This study investigated the influence of peroral supplementation of selenium at a dose of 0.3 mg Se.kg^{-1} feed dry matter (DM) in the form of Na_2SeO_3 and vitamin E 300 mg.ml^{-1} added to water at a rate of 4 ml.l^{-1} during 60 days. The supplemented group comprised 14 pigeons and their results were compared with a control group of 14 pigeons fed non-supplemented commercial feed. Blood samples were collected and examined at the beginning of the supplementation period, one day before a 300 km race and after the race. Pigeons from the supplemented group exhibited increased plasma levels of Se and vitamin E, as well as the activity of glutathione peroxidase (GSH-Px) before and after the 300 km race in comparison with the controls. Comparison of the activity of the enzymes: alanine aminotransferase (ALT), aspartate aminotransferase (ASP), alkaline phosphatase (ALP), and creatinine kinase (CK) at the beginning of the supplementation and one day before the race showed no changes. A significant ($P < 0.05$) increase in the activity of all investigated enzymes were observed after the race in both groups.

Key words: glutathione peroxidase; racing pigeons; selenium; supplementation, vitamin E

INTRODUCTION

Pigeons belong among the first animals to be domesticated and are strongly attached to the place where they live. Since the oldest times, pigeons attracted the interest of people by their unbelievable sense of spatial orientation, flying capabilities, monogamous behaviour and bond to their habitat. For many people pigeon keeping became a passion, a hobby and meaningful passing of free time [3]. By successfully returning from the race in a winning position, the

pigeons demonstrate favourable factors, such as their inherent qualities, health state, stress related to transport and hygiene conditions in their breeding environment. Shortened flying times at competitions and more races per season indicate the higher performance of these birds which is reflected in their health and short periods of regeneration. The increasing stress is associated with weakened immunity, higher susceptibility to diseases and the absence of their expected performance [4].

Today it is not possible to rely on the quality of pigeons, their health and "functioning" of their inherent traits. Bringing them into form at the right time and appropriate knowledge of metabolic and antioxidative processes which take place in their bodies while exposed to extreme demands help the breeders to select suitable individuals which can be subjected to intensive training and perform to expectations [9].

The aim of this study was to investigate the influence of peroral supplementation of Se and vitamin E (vit E) on racing pigeons during a 300 km race with respect to serum concentrations of Se, vitamin E and activity of glutathione peroxidase (GSH-Px), alanine aminotransferase (ALT), aspartate aminotransferase (ASP), alkaline phosphatase (ALP), and creatinine kinase (CK) in the blood.

MATERIALS AND METHODS

Breeding establishment and training of pigeons

The pigeons were kept in a wooden pigeon house of the garden type with dimensions of 7.5 m × 2.5 m × 2 m, intended for housing 60 racing pigeons (*Columba livia*), divided by wirework into two sections, for pigeons and doves. In each section there were 16 boxes, each with sliding floor grill, and a drinker and a feeder. During the racing season (May – July), the pigeons took part in 19 races, ranging according to the racing distance from less demanding (120 km) to very demanding (1400 km).

Feeding and selection of pigeons

During the racing season the pigeons were fed a commercial mixed feed intended for racing birds in the amount of 20 g/head, in the morning and in the evening. After returning from races they were given post-racing mixed feed (Tab. 1) at a dose of 35 g/head together with glucose and electrolyte (Dacovit Dextrose 600, Merca Systems, UK) added to water at a dose of 10 g.l^{-1}. On the following day, after the morning training, the feed was supplemented with probiotic (Propigeonv plv. 1000, Inproco) at a dose of 0.5 g/head for 3 days.

Sixty days before the planned 300 km race, we randomly selected 14 pigeons (7 males and 7 females, 2—4 years old) which were placed in a separate section of the pigeon house and received feed supplemented with 0.3 mg Na_2SeO_3.kg^{-1} feed dry matter (DM) and Hydrovit E forte 300 mg.ml^{-1} (PharmaGal, SR) in water at a dose of 4 ml.l^{-1}. A group of similar composition (7 males and 7 females, 2—4 years old) was randomly formed from non-supplemented pigeons and served as a control.

Sampling and analysis of samples

Before the onset of the supplementation, one day before the race and after the return of the pigeons from the race, 3 ml of blood was withdrawn from the vena femoralis of the birds from the experimental group (supplemented with sodium selenite and vitamin E) and from the control group. All samples were stored at –54 °C up until the analysis. The concentration of Se in the blood plasma was determined using a fluorometric method of Rodriguez et al. [10]. The activity of GSH-Px in the blood was determined by means of a Glutathione peroxidase assay kit (Randox-Ransel, UK) according to Paglia and Valentine [7]. The samples of mixed feed before and after addition of 0.3 mg Na_2SeO_3.kg^{-1} feed DM were subjected to wet mineralization in a microwave module LS 1200 (Milestone, USA) and analysed for the content of Se by an atomic absorption spectrometer Zeman 4100 (Perkin Elmer, USA), using procedure according to Pechová et al. [8]. The vitamin E (α-tocopherol) in the blood plasma was determined by a HPLC method [6]. Homogenized samples of mixed feed were subjected to saponification and extraction and analysed for the content of α-tocopherol by HPLC method according to ES No. 152/2009 [5]. The determinations of the enzymatic activities of ALT, AST, ALP and CK were carried out by diagnostic test kits BIO-LA-TEST (Erba Lachema, Brno, CR).

Statistical analysis

The values of Se and α-tocopherol, as well as enzymatic activities of GSH-Px, ALT, AST, ALP and CK were processed statistically by a non-paired T-test by comparing the results from the supplemented group with those from the control. The values measured in each group after individual

samplings were compared by post Tukey test using $P < 0.05$ as the level of significance. The results are presented as the means (M) ± standard deviation (SD).

RESULTS AND DISCUSSION

All tissues possess an antioxidant system which protects them against damage to biologically important molecules by free radicals and eliminates hydrogen peroxide. Antioxidants are involved in these processes as they are able to deactivate the charged particles of free radicals and to eliminate them before they initiate further chain destruction. Selenium compounds and vitamin E belong among the most effective antioxidants, however, they are frequently deficient in commercially mixed feeds [13].

Table 1. Composition of mixed feed

Racing diet[1]		Post-racing diet[1]	
Component	**%**	**Component**	**%**
Maze (2 varieties)	30	Dari (sorghum)	20
Wheat	23	Barley	20
Barley	8	Kardi (safflower)	15
Sunflower (2 varieties)	10	Shelled rice	10
Peas	6	Paddy rice	9
Field pea	6	Wheat	9
Shelled oats	5	Milo (sorghum)	7
Dari (sorghum)	4	Hemp	3
Millet	4	Linseed	3
Rape	2	Oat	2
Vetch	2	Vetch	2
Se [μg.kg^{-1} DM]	**93**		81
Se* [μg.kg^{-1} DM]	**386**		365
Vitamin E [IU]	**48**		40

Se* — selenium content after addition of 0.3 mg Na_2SeO_3.kg^{-1} DM; [1]Racing and Post-racing mixed feed purchased from Röhnfried, GmbH & Co KG, Netherland

Selenium, primarily known for its antioxidant, anti-inflammatory and antiviral properties, is an essential trace mineral for normal growth and development of livestock and humans. Selenium exerts its biological functions as a component of at least three groups of proteins: glutathione peroxidase that is responsible for the reduction of hydroperoxides in cells, plasma and gastrointestinal tract; the iodothyronine deiodinases, responsible for the peripheral deiodinates of thyroxin (T3) to 3,5,3' triiodothyronine (T2) and other metabolites; and thioredoxin reductases, which are involved in many cell functions, including the control of apoptosis and maintenance of the cellular redox state [11].

Vitamin E, as an antioxidant with a lipophilic structure, accumulates in extra- and intra-cellular cell membranes, chylomicrons, adipose tissue and the liver, where it removes free radicals produced in the lipid peroxidation reaction and thus protects membrane-bound polyenic acids against peroxidation [1, 2].

Table 2 presents a comparison of plasma levels of Se and vitamin E in supplemented and control groups of pigeons. At the beginning of supplementation period, no differences were observed in the investigated parameters. Increased plasma levels of Se and vitamin E were recorded in supplemented pigeons one day before the race as well as after return from the race in comparison with control birds.

Table 2. Comparison of plasma concentration of Se and vitamin E in pigeons

Parameter	Sampling	Supplemented group (n = 14)	Control group (n = 14)	P
		M ± SD	M ± SD	
Se (μg.l–1)	Onset of suppl.	62.4 ± 4.7^{a}	63.1 ± 3.6	NS
	Before race	124.5 ± 5.4^{b}	77.3 ± 3.6	(P < 0.001)
	After race	110,7 ± 6,1^{b}	71.6 ± 4.4	(P < 0.001)
Vitamin E μg.ml–1	Onset of suppl.	4.05 ± 0.34^{a}	4.32 ± 0.22^{a}	NS
	Before race	8.33 ± 0.47^{d}	5.87 ± 0.35^{c}	(P < 0.01)
	After race	5.66 ± 0.22^{a}	4.01 ± 0.46^{a}	(P < 0.05)

M — mean; SD — standard deviation; P – statistical significance; NS —non significant; means with different superscript letters differ significantly: a, b — P < 0.05; a, c — P < 0.01; a, d — P < 0.001

Table 3. Enzymatic activity during the supplementation period

Parameter	Sampling	Supplemented group (n = 14)	Control group (n = 14)	P
		M ± SD	M ± SD	
GSH-Px [IU.g^{-1} Hb]	Onset of suppl.	124.3 ± 9.7a	118.5 ± 9.2a	NS
	Before race	291.6 ± 19.2c	209.1 ± 15.4c	P < 0.05
	After race	320.4 ± 21.7d	232.7 ± 24.2d	P < 0.01
ALP [μkat.l^{-1}]	Onset of suppl.	9.33 ± 5.64a	8.13 ± 3.41a	NS
	Before race	11.30 ± 4.17a	10.16 ± 5.20a	NS
	After race	28.82 ± 9.13b	33.97 ± 9.16d	NS
AST [μkat.l^{-1}]	Onset of suppl.	3.2 ± 0.93a	3.8 ± 0.78a	NS
	Before race	3.7 ± 0.82a	3.4 ± 1.05a	NS
	After race	6.7 ± 2.10c	8.9 ± 2.74d	NS
ALT [μkat.l^{-1}]	Onset of suppl.	0.48 ± 0.11a	0.38 ± 0.09a	NS
	Before race	0.41 ± 0.12a	0.56 ± 0.11a	NS
	After race	0.95 ± 0.22c	1.27 ± 0.37d	NS
CK [IU.l^{-1}]	Onset of suppl.	127.2 ± 22.1a	141.6 ± 16.4a	NS
	Before race	156.7 ± 31.3a	178.3 ± 37.1a	NS
	After race	305.4 ± 49.2d	347.2 ± 53.6d	NS

M — mean; SD — standard deviation; P — statistical significance; NS — non significant; means with different superscript letters differ significantly: a, b — P < 0.05; a, c — P < 0.01; a, d — P < 0.001

Schoonheer et al. [12] reported increased number of hydroxide radicals during 2 hour flight simulation in carrier pigeons. The authors also observed more rapid degradation of these radicals, as well as increased activity of GSH-Px in the group of pigeons supplemented with 195 μg Se.kg^{-1} feed, compared to pigeons supplied a common mixed feed (30 μg Se.kg^{-1} feed). Our study confirmed an increased activity of GSH-Px in the supplemented group of pigeons before the race, as well as after their return from the race in comparison with control birds. Table 3 presents the activity of the investigated enzymes during the period of SE and vitamin E supplementation. No significant differences in the activity of ALT, ASP, ALT and CK between experimental and control groups were observed at the onset of supplementation or one day before the race. An increased activity ($P < 0.05$) of all investigated enzymes was observed after the race in both groups.

CONCLUSIONS

Peroral supplementation of Se and vitamin E resulted in higher levels of these antioxidants in the plasma as well as in increased activity of GSH-Px one day before the race and after the race in comparison with the control. The increased activity of GSH-Px can be explained by the physical muscle activity and the elimination of free radicals. After the race, pigeons from both groups exhibited decreased concentrations of α-tocopherol, probably due to its increased consumption related to physical exertion. The mineral-vitamin supplementation was not reflected in the biochemical enzymatic parameters of ALP, AST, ALT and CK, the activity of which is associated with physical load. The supplementation of preparations based on Se and vitamin E can provide effective antioxidant protection and thus contribute to better performance and rapid recovery of individual birds following their flying efforts.

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REFERENCE VALUES FOR THE OPHTHALMIC SCHIRMER TEAR TEST AND THE INTRAOCULAR PRESSURE IN HEALTHY CHINCHILLAS

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ABSTRACT

The objective of this study was to measure the intraocular pressure (IOP) and tear production before and after topical anaesthesia in healthy chinchillas (*Chinchilla lanigera*). Thirteen healthy non-sedated chinchillas (eight males and five females) were used in this study. The tear production was measured by the novel endodontic paper point tear test (PPTT) using Roeko Colour No. 30 Paper points. Following the PPTT, one drop of 0.4 % oxybuprokainium chloride was added to the eye to anaesthetise the cornea and the IOP was measured using the Tono-Pen Avia®Vet. Excess anaesthetic was removed from the conjunctival fornix using a sterile cotton tipped applicator and the PPTT II was performed. The PPTT I and II were measured in 26 eyes, mean ± standard deviations (SD) were 7.98 ± 1.95 mm.min^{-1}, and 9.71 ± 3.52 mm.min^{-1} respectively. The IOP was measured in 20 eyes, and the mean ± SD was 28.52 ± 12.48 mmHg (35.50 ± 9.31 mmHg in males and 21.53 ± 11.57 mmHg in females). There was no significant difference in the PPTT results between the left and right eyes or between the male and female groups. The males were found to have a significantly higher IOP than females and the PPTT II was significantly greater than the PPTT I. The PPTT test proved to be effective, easy to use, and reliable, causing little apparent discomfort to the chinchillas and could prove to be a much more effective tool than the Schirmer tear test for the evaluation of the tear production in animals with small eyes and/or low aqueous tear production. The mean intraocular pressure proved to be much higher in this population of chinchillas than those previously studied and so further investigation is warranted before a reliable reference range may be produced.

Key words: eye; healthy chinchilla; intraocular pressure; Schirmer tear test

INTRODUCTION

Chinchillas are particularly predisposed to developing keratoconjunctivitis sicca due to their relatively large and prominent corneas, low blink frequency, and exposure

to a potentially dusty environment from low quality dust baths or poor hygienic conditions [8, 10]. It is, therefore, essential that normal tear production values are known and that keratoconjunctivitis sicca can be diagnosed promptly. The Schirmer tear test (STT) has previously been used to assess the aqueous tear production in chinchillas, however, the results were difficult to interpret due to the very low amount of tears produced with a mean ± standard deviation (SD) of 1.07 ± 0.54 mm.min^{-1}. The tear level sometimes did not even reach the notch in the test strip [7, 11]. The phenol red thread test was also used to assess the aqueous tear production in chinchillas and it produced more readable results with a mean ± SD of 14.6 ± 3.5 mm.15 s^{-1} [11]. A more accurate and reliable method of tear detection in this species and in other animals with small palpebral fissure lengths and low aqueous tear production, would be of great benefit. Several studies have used the novel endodontic paper point tear test (PPTT) in animals with small palpebral fissure lengths to assess the aqueous tear production with promising results [4, 5, 6]. In our study we assessed the suitability, reliability and ease of use of the novel endodontic paper point tear test (PPTT), and established a reference range as a basis for further investigations into this promising new test method.

There are few reports in the literature of raised intraocular pressure (IOP) due to primary glaucoma in chinchillas, however, luxation of the lens, cataracts and lens induced uveitis have been reported to cause secondary glaucoma [3, 8, 15]. Only three papers have looked into the normal physiology of chinchilla eyes and a different tonometer was used in each study, so the reference ranges are not directly comparable, making interpretation of what is a normal IOP more difficult [7, 11, 12]. The aim of our study was to measure the IOP using an applanation tonometer and to add this data to that of the current literature, in order to create a more reliable reference range and enable the clinician to better interpret IOP values obtained during clinical examinations of the chinchilla.

MATERIALS AND METHODS

Thirteen healthy chinchillas, eight males and five females, were brought into the clinic. They ranged from 1.5 to 6.5 years of age and were from 600—900 g in weight. All 13 chinchillas were tested for tear production and ten of them were tested for their IOP. The chinchillas had no obvious eye pathologies and were in good general health. They were tested in groups of three or four and the procedures were carried out at approximately the same time in the morning, generally between 08 : 30 and 09 : 30 a.m. The same experienced person restrained the chinchillas each time, using a towel, with one hand around the thorax and one supporting the lower body. The same restraint was used for all tests and they were all performed by the same experienced person. The paper point tear test was measured initially, after which, one drop of anaesthetic was placed into each eye; the chinchilla was then put back whilst the next chinchilla went through the same procedure. Once the last chinchilla received the anaesthetic, the first was brought out again, restrained, the IOP measured and then the PPTT II performed.

Roeko Colour No. 30 endodontic paper points (Coltene, Altstätten, Switzerland) were used for this test; a cm ruler was necessary for the measurement of the wetted point and a stopwatch was used to time the test accurately. This specific type of paper point was chosen as it was used in previous studies of tear secretion in rabbits, black tufted marmosets, rats and mice [4, 5, 6].

The paper point was placed into the lower fornix of the left eye, the stopwatch started and a few seconds later, a second paper point was placed in the right eye taking note of the time of insertion according to the stopwatch. The paper points were then removed in turn, after each one had been in placed for exactly one minute. The tear uptake was immediately measured by lightly pressing the inserted end onto a ruler, causing the point to bend where the tear level reached. The length of the wetted strip was then read off the ruler in millimetres and recorded.

One drop of 0.4 % oxybuprokainium chloride (Benoxi, Unimed Pharma) was administered to each eye and left to work for at least ten minutes. The IOP was measured using the Tonopen Avia®Vet™ (Reichert, New York, USA); three measurements were taken and averaged for each eye. Following the IOP testing, any excess tears were removed using a sterile cotton tipped applicator and the PPTT II was measured in the same manner as previously described for the PPTT I.

The Wilcoxon Signed Ranks Test was used to compare the left and right eyes, and the PPTT I and II values. The Mann Whitney U test was used to compare the males and females. The Spearman's rank-order correlation test was

calculated to investigate any linear correlation between the ages and the three ophthalmologic parameters. The data were considered to be statistically significant when $P < 0.05$, in which case, the null hypothesis would be rejected. The results were expressed as the mean ± SD.

RESULTS

All results are presented in Tables 1—4. Table 1 shows the overall mean ± SD for all three tests from both eyes of each chinchilla. Table 2 shows the confidence interval and overall range of results recorded for all three tests. Table 3 and 4 compared the mean ± SD of the left and right eyes and the male and female chinchillas respectively.

After the statistical analysis it was found that there was no difference in the PPTT I between the left and right eyes ($P = 0.48$) or between the males and females ($P = 0.09$) and there was no correlation between the PPTT I and age ($P = 0.61$). There was also no difference in the IOP between the left and right eyes ($P = 0.26$) but there was a significant difference in the IOP between the males and females ($P = 0.01$) with the males showing a higher mean IOP (Table 4). There was no difference in the PPTT II between the left and right eyes ($P = 0.58$) or between the males and females ($P = 0.20$) and there was no correlation between the PPTT II and age ($P = 0.15$). The PPTT II was, on average, 22 % higher than the PPTT I (Table 1) and this difference was found to be significant ($P = 0.03$).

Table 1. Paper point tear tests and the intraocular pressure results

	PPTT I [mm.min^{-1}]	IOP [mmHg]	PPTT II [mm.min^{-1}]
Overall Mean ± SD	7.98 ± 1.95	28.52 ± 12.48	9.71 ± 3.52

Table 2. Paper point tear tests and the intraocular pressure confidence intervals and reference ranges

	PPTT 1 [mm.min^{-1}]	IOP [mmHg]	PPTT II [mm.min^{-1}]
95 % CI of the Mean Range	7.19—8.77	22. 68—34.36	8.29—11.13
	5.0—11.5	5.00—49.67	4.0—16.0

Table 3. Paper point tear tests and the intraocular pressure in the left and right eyes

	PPTT 1 [mm.min^{-1}]	IOP [mmHg]	PPTT II [mm.min^{-1}]
Left eye (Mean ± SD)	8.23 ± 2.17	30.07 ± 12.84	9.12 ± 3.59
Right eye (Mean ± SD)	7.73 ± 1.76	26.97 ± 12.60	10.31 ± 3.49

Table 4. Paper point tear tests and the intraocular pressure

	PPTT 1 [mm.min^{-1}]	IOP [mmHg]	PPTT II [mm.min^{-1}]
Male (Mean ± SD)	8.5 ± 1.9	35.50 ± 9.31	10.4 ± 3.1
Female (Mean ± SD)	7.2 ± 1.7	21.53 ± 11.57	8.7 ± 4.0

DISCUSSION

The paper point tear test proved to be accurate and easy to perform, allowing both eyes to be measured almost simultaneously, and caused minimal discomfort to the chinchillas. The rigidity of the paper point allowed it to be easily inserted into the eye and with one or two exceptions, it stayed in place for the entire minute. The paper point tear test proved to be a useful method of measurement of the aqueous tear production in the chinchillas. It seems to be more reliable than the modified Schirmer tear test, as each paper point is exactly the same and it is easier to read and interpret than the STT when aqueous tear volumes are low.

Our data were in a similar range to that of the PPTT in other small mammals, with a higher mean than rats (6.18 ± 2.06 mm.min^{-1}) and mice (4.39 ± 1.45 mm.min^{-1}), and a lower mean than rabbits (13.8 ± 1.5 mm.min^{-1}) [4, 5, 6]. This is to be expected considering the smaller size of the mice and rats and the higher STT values for rabbits in the literature (5.3 ± 2.96 mm.min^{-1}) [1, 2].

The PPTT II determines the basal tear secretion, and is compared with the reflex secretion measured by the PPTT I. In our study, the mean PPTT II was higher than

the mean PPTT I which was contrary to what would be expected. This may have been caused by the process of wiping the excess anaesthetic out of the eye; the same problem that Weiser et al. [14] found when they swabbed the conjunctival sac in the cow. They solved this by waiting a few minutes before performing the test in order to allow the excess anaesthetic to clear naturally. In chinchillas this may not work as well because of their low blink frequency. In our study, there was a delay between adding the anaesthetic and carrying out the test, however, the IOP was measured and the conjunctival sac was dried just prior to the PPTT II test which may have caused some reflex tearing due to the stimulation of structures other than the cornea. These results may also be due to a low corneal sensitivity in chinchillas or a reduction or the absence of reflex tearing in this species. However, Muller et al. [11] found that chinchillas have a low corneal touch threshold (mean 1.5 ± 0.9 g. mm^{-2}); lower than that of the guinea pig (3.7 g.mm^{-2}), indicating that corneal sensitivity is similar to other species, for example the horse which has a corneal touch threshold of 1.23 ± 1.07 g.mm^{-2} [13, 14].

The Tono-Pen applanation tonometer produced some quite variable results with a coefficient of variance of 44 % which is relatively high. The readings obtained by the applanation tonometer are very reliant on the individual operator's technique, as the instrument needs to be gently tapped on the cornea several times for a reading to be taken which leads to a certain level of inherent variability. The level of stress in the animal tested and the amount of pressure applied to the neck region in a struggling animal, for example may also have affected the results [9]. The two studies that used applanation tonometers to test the IOP in the chinchillas found a mean ± SD of 18.5 ± 5.75 mmHg using the Mackay-Marg tonometer, and 17.71 ± 4.17 mmHg using the Tono-Pen XL. The results we obtained were considerably higher with a much larger variability [2, 7]. The chinchillas were all healthy with no evidence of ocular pathologies so the variability was unlikely to be due to disease or biological variations. More likely, the causes of the variability would be differences in the restraint pressure, especially in the more excitable chinchillas, operator technique, or an increase in stress causing elevated intraocular pressure [9]. A significant difference was found in our study between the mean IOP in the males and females, with the male group showing a larger mean IOP than the female group. Looking at just the female chinchillas, the mean IOP was much closer to what would be expected in the chinchilla based on the current literature. It is possible that the males used in this study were more stressed than the females, leading to a higher IOP. The sample size in our study, however, was too small to make a definitive conclusion regarding the difference in the IOP between the genders but it would be an interesting area for future investigations.

CONCLUSIONS

The novel endodontic paper point test proved to be very easy to use, the variability of the data was low and reflex tearing in the chinchillas appeared to be minimal. Further studies using this method should be carried out in order to verify the reproducibility of the tests. The intraocular pressure was found to be much higher than that recorded in previous studies and showed a large variability, most likely caused by operator techniques and higher stress levels in the male chinchillas. Further studies should be carried out on these chinchillas to determine if the results were aberrant due to external influences or if these chinchillas naturally have a higher IOP. In the clinical situation it is likely that stressed and excitable animals will be presented to the clinician, so these results, although out of the normal range so far established, are still useful as they reflect values that could be found in a clinical setting. This variability highlights the importance of taking multiple IOP readings in a patient to avoid a false diagnosis of glaucoma.

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STUDY OF ANTIOXIDANT EFFECTS OF SELECTED TYPES OF COFFEE

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ABSTRACT

Coffee is a rich source of dietary antioxidants which protects the human body against the effects of dangerous free radicals. The aim of this study was to determine and compare the antioxidant activity, content of total phenols and flavonoids in selected types of coffee with respect to the way of their processing. The individual coffees were investigated with regard to their origin and composition. The antioxidant effects were determined by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay. The content of total phenols was analysed by the Folin-Ciocalteu method and the content of flavonoids in the coffee extracts was determined by a colorimetric method. The highest antioxidant activity was exhibited by the extract of unroasted ground 100 % green coffee *Arabica* (89.55 %), and the high scavenging of free radicals was achieved also by the extracts of roasted ground 100 % coffees *Arabica* and *Robusta*. The highest levels of total phenols (77.54 µg.ml^{-1}) and flavonoids (1.74 µg.ml^{-1}) were measured in the extract of unroasted ground 100 % green coffee *Arabica*. High levels of these substances were found also in extracts of roasted ground 100 % coffees *Arabica* and *Robusta*. The lowest levels of total phenols (31.24 µg.ml^{-1}) and flavonoids (0.91 µg.ml^{-1}) were detected in the extract of instant coffee *Arabica*. The processing of coffee by roasting decreased the level of the investigated antioxidant components but considerably improved the taste and aroma, the properties that make coffee one of the most popular drinks in the world.

Key words: antioxidant effect; *Coffee Arabica* (L.); free radicals

INTRODUCTION

The human body is constantly exposed to the effects of free radicals produced by various metabolic pathways. They get into the bodies through air, liquids and food. They are dangerous due to their ability: to attack cell structures, inhibit their normal functions, and thus contribute to the development of numerous diseases. Antioxidants play an important role in the battle against the undesirable effects

of free radicals. The contribution of antioxidants is extensive; they protect human bodies against premature ageing, weakening of the immune system, and other health problems caused by free radicals. The human body is capable of naturally producing its own effective antioxidants but their amount is not always sufficient and thus it is necessary to take them in the food. They are found, for example in fruits, vegetables, chocolate, red wine and cereals. Recently, increased attention has been paid to the beneficial effect of coffee on human organism. Coffee is also an excellent source of antioxidants, particularly polyphenol compounds, which participate considerably in the neutralisation of free radicals [9].

Green (raw) coffee beans are the richest source of chlorogenic acids, one of the strong antioxidants with a positive influence on health. Research has demonstrated that these acids can function as an inhibitor of glucose-6-phosphatase. Owing to chlorogenic acids, the body compensates a decrease in the level of blood glucose by the break-down of fats, and thus, green coffee is presently much sought-after for the preparation of weight reduction. However, chlorogenic acids are thermally unstable and thus undergo many changes during roasting. Roasting is, however, an inevitable way of processing of green coffee as it gives complex aroma and taste to the final product (the properties required by coffee consumers). The degree and conditions of roasting considerably affect the volatile components in coffee. Aroma of light roasted coffee differs considerably from that of dark roasted coffee [10]. Owing to its pleasant aroma, taste and stimulating effect on the body and mind, coffee is one of the most popular drinks in the world.

The aim of this study was to determine the antioxidant properties and content of flavonoids and total phenols in roasted and unroasted coffee varieties *Coffea Robusta* and *Coffea Arabica* by means of the spectrophotometric methods.

MATERIALS AND METHODS

For analysis, we purchased from a store chain, 7 coffee types (3 roasted and ground, 2 instant and one unroasted green coffee) of varieties *Coffea Robusta* and *Coffea Arabica*. The list of coffee types is presented in Table 1. For preparation of the extracts, we used 5 g quantities of ground coffee and 2 g of instant coffee. The extracts were prepared by pouring boiling water (100 °C) over coffee samples and after 10 minutes, filtering the extracts and using them for the ultimate determination. All chemicals used in the experiments were supplied by Sigma Aldrich (Germany).

Table 1. Coffee types used for the determination of antioxidant activity

Sample No.	Coffee type
1	*Arabica*: roasted ground 100 % coffee (Gold)
2	*Arabica*: roasted ground 100 % coffee (Extra special)
3	*Robusta*: roasted ground 100 % coffee
4	*Robusta*: roasted ground coffee
5	*Arabica*: Instant 100 % coffee
6	*Arabica*: Instant coffee (Crema Gold)
7	*Arabica*: unroasted ground 100 % green coffee

Spectrophotometric determination of antioxidant activity by the DPPH radical scavenging assay

The antioxidant properties were determined by the method of Heilerová et al. [6]. The principle consists in the reaction of the coffee extract with a stable 2,2-diphenyl-1-picryl-hydrazyl (DPPH). The ability of coffee extract to scavenge free radical is directly related to the rate and extent of decolouration of the synthetic radical DPPH and at the same time as a decrease in the absorbance. The antioxidant activity of coffee extracts was calculated as a per cent of inhibition of the DPPH radical. The procedure was as follows: 3.9 ml aliquot of the stock solution of DPPH (0.0035 g per 100 ml methanol) was pipetted into a cuvette and the absorbance (A0) of this solution was measured at 515 nm. Subsequently, we prepared a reaction mixture by adding 100 µl aliquot of coffee extract. After mixing and incubation for 5 minutes, the absorbance of the reaction mixture (AA) was measured. The antioxidant activity (per cent of DPPH radical inhibition) was calculated according to the formula: % inhibition = $[(A0 - AA)/A0] \times 100$.

Spectrophotometric determination of the total phenols

The content of the total phenols in the coffee extracts was determined by the Folin-Ciocalteu method described in the study by Singleton et al. [12]. The method is

based on the oxidation-reduction reaction during which the phenol compounds are oxidised at a parallel reduction of Folin-Ciocalteu (FC) reagent and the development of a blue colour. The intensity of colouring correlates with the redox properties of phenolic compounds present in coffee extracts. The procedure was as follows: coffee extracts and the FC reagent were diluted with distilled water 1:1000 and 1 : 10, respectively. Then, 1 ml of the diluted coffee extract was pipetted into a test tube and 5 ml of diluted FC-reagent and 4 ml Na_2CO_3 (75 g.l^{-1}) were added and the content was mixed. After a 30 minute incubation at room temperature, the absorbance of the solutions were measured at a wavelength of 765 nm, against a blank which contained distilled water instead of the coffee extract. Gallic acid in concentrations of 0.038—0.3 mg.l^{-1} was used as a standard. Gallic acid absorbance ranges from 0.05 to 0.555 nm.

Determination of total flavonoids

The content of total flavonoids was determined by the colorimetric method described in the study by Kim et al. [7]. The addition of an aluminium chloride solution to flavonoids results in the formation of yellow chelate complexes. The content of flavonoids was determined spectrophotometrically by measuring the intensity of the yellow colour. The procedure was as follows: coffee extracts were diluted with distilled water 1 : 100. To 1 ml of the diluted coffee extract, we added 4 ml of distilled water and 0.3 ml $NaNO_2$ (50 g.l^{-1}). After 5 min of incubation, we added 0.3 ml AlCl3 (100 g.l^{-1}), and after an additional 6 minutes, 2 ml NaOH (1 mol.l^{-1}) and 2.4 ml distilled water, mixed the content and measured the absorbance of the samples (510 nm) against a blank which contained distilled water instead of coffee extract. The content of total flavonoids was determined using the quercetin standard (0.05—0.4 mg.l^{-1}).

Processing and presentation of the results

The results of the determinations conducted in individual coffee extracts are presented as arithmetic means (x) and standard deviations (± SD). All measurements were carried out in triplicate.

RESULTS

Of all tested coffee types, the extract of unroasted ground coffee exhibited the highest capacity (Sample 7; 89.55 %) for scavenging DPPH radicals. High antioxidant activity was determined also with extract of roasted ground 100 % *Arabica* — Sample 1 (82.5 %). The lowest percentage of free radical scavenging was observed with extract of instant coffee with high content of coffee variety *Arabica* — Sample 6 (56.16 %). Similar to antioxidant activity, the extract of unroasted ground coffee exhibited the highest content of total phenols (7754 µg.ml^{-1}) followed by extract of roasted ground 100 % *Arabica* — Sample 1 (73.64 µg. ml^{-1}). The level of total phenols was the lowest in Sample 6 (31.24 µg.ml^{-1}); the extract of instant coffee with high content of coffee variety *Arabica*. The sample 7 rated the best with regard to total flavonoids (1.74 µg.ml^{-1}). The level of total flavonoids in the extracts of the remaining types of coffee was above 1 µg.ml^{-1} with the exception of Sample 6, instant coffee with high content of variety *Arabica*, where it reached (0.91 µg.ml^{-1}). The mean levels of the values and standard deviations are presented in Table 2.

DISCUSSION

Coffee is the main source of polyphenolic compounds which are well known for their antioxidant effects. They contain chlorogenic acids with biological effects mostly related to their remarkable antioxidant, anti-mutagenic, anti-carcinogenic and anti-inflammatory activities. The chlorogenic acids exhibit a high capacity to scavenge reactive oxygen radicals. These polyphenols are able to inhibit inflammatory processes and propagation of tumours by means of the deactivation of pro-oxidative enzymes [5]. Some studies demonstrated that green coffee extracts have; anti-hypertension effects [8], inhibit the accumulation of fats and body weight gain [4] and modulate glucose metabolism in humans [1]. These biological effects were ascribed to the chlorogenic acids present in green coffee.

The studies involved in the determination of the antioxidant capacity of coffee of a variety of *Arabica* showed that the content of total phenols in roasted coffee is lower than in unroasted green coffee [3], which correlates with our results. Lower levels of polyphenols in roasted coffee can be ascribed to their polymerization, autoxidation or degradation during roasting [2]. The content of total phenols determined in our study correlates with the results of Cheong et al. [3]. The highest antioxidant activity was observed in the extract of green unroasted 100 % coffee

Table 2. Antioxidant capacity, total phenols and flavonoids in the types of coffee (mean ± SD)

Sample	Antioxidant capacity [%]	Total phenols [μg.ml^{-1}]	Flavonoids [μg.ml^{-1}]
1	82.5 ± 1.62	73.64 ±14.92	1.43 ± 0.18
2	78.92 ± 2.46	55.7 ± 6.11	1.05 ± 0.04
3	78.46 ± 0.66	51.37 ± 18.4	1.14 ± 0.08
4	79.93 ±1.49	59.9 ± 11.03	1.41 ± 0.05
5	71.06 ± 1.20	57.47 ± 11.24	1.02 ± 0.05
6	56.16 ± 4.09	31.24 ± 18.07	0.91 ± 0.01
7	89.55 ± 0.37	77.54 ± 15.36	1.74 ± 0.03

SD — standard deviation

Arabica. This coffee extract also showed the highest content of total phenols and flavonoids. The lowest antioxidant capacity was measured in the extract of 100 % coffee *Robusta*. Extracts of roasted ground coffees exhibited a comparable capacity to scavenge free radicals. This capacity correlated with the levels of total phenols and flavonoids. Relatively lower levels of total phenols and flavonoids were recorded in extracts of instant coffee. These samples exhibited also lower antioxidant capacity. The results of our study agree with the investigations of Ramalakshmi and Rao [12] who reported that extracts of green coffee contain higher levels of polyphenols and chlorogenic acids than extracts of instant coffee. The relatively low levels of total phenols can be explained by the degradation of these compounds during roasting and their release to water during extract preparation.

CONCLUSIONS

The best antioxidant properties and the highest levels of total phenols and flavonoids were detected in ground green coffee *Arabica* and the lowest in the extract of instant coffee with high content of variety *Arabica*. These results allowed us to conclude that the antioxidant activity of coffee decreases with roasting, but coffee still retains considerable capacity to scavenge free radicals and sufficient level of total phenols and flavonoids which also act as antioxidants.

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EPIZOOTIOLOGICAL STUDY OF THE OCCURRENCE OF CANINE BABESIOSISIN SOUTHWESTERN SLOVAKIA

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ABSTRACT

This epizootiological study was carried out to investigate the occurrence of canine babesiosis in southwestern Slovakia. The study focused on the proportion of the species of ticks serving as babesia vectors of babesiosis in the ticks collected from selected locations close to Komárno, in southwestern Slovakia. Additionally, observations were made on the health and overall clinical signs in dogs suspected of having babesiosis. In 2014 we collected ticks from vegetation by the drag cloth (flagging) method and also directly from dogs. A totally of 622 ticks were collected: 491 *Dermacentor reticulatus* and 131 *Ixodes ricinus*. Representative samples of ticks (n = 103) were examined by the molecular method and the presence of *Babesia* spp. DNA was identified in 12.5 % of the *Ixodes ricinus* ticks collected by flagging and in 9.5 % of the *Ixodes ricinus* ticks collected from the dogs. *Babesia canis* (KU681325) with 90 bp sequence, 100 % identical with *Babesia canis* isolates from dogs for example from: Turkey (KF499115), Rumania (HQ662634), Croatia (FJ209025), Poland (EU622792) and Russia (AY962186), was confirmed after sequencing in one *Ixodes ricinus* female obtained from a dog. This was the first confirmation of the occurrence of *B. canis* in *Ixodes ricinus* ticks in Slovakia. In 2.2 % of the *Dermacentor reticulatus* ticks obtained from vegetation by flagging, we were able to diagnose the DNA of *Babesia canis*. In 4.8 % of the *Dermacentor reticulatus* ticks collected from dogs, the presence of *Babesia* spp. was confirmed. Thirty three dogs with suspicion of babesiosis were observed in an ambulance by their health and clinical signs. The loss of appetite was observed in 22 patients (66.7 %), apathy in 19 cases (57.6 %), and fever in 19 cases. Closer specification indicating babesiosis was finding engorged ticks on the dog bodies which occurred in 21 cases (63.6 %), haematuria in 8 cases (24.2 %), anaemia in 4 cases (12.1 %), and tremor in 6 cases (18.2 %). Other non-specific signs, such as diarrhoea, vomitus, slowed-down movement, ataxia and lacrimation were also observed in less than 18 % of the examined dogs. On the basis of the specific clinical signs, blood was withdrawn from 33 dogs for preparation of blood smears and in 19 of them (57.6 %) babesia in erythrocytes were confirmed microscopically.

Key words: *Babesia*; *Dermacentor marginatus*; dog; epizootiology; *Ixodes ricinus*; PCR; prevalence

INTRODUCTION

During the past two decades, the number of tick-borne pathogens has increased and currently ticks raise serious health problems in many European regions. Due to climate changes which affect the biology of these vectors, they are spreading tick-borne diseases. Babesiosis is a protozoan disease with a worldwide distribution which affects particularly the blood and reticuloendothelial system of mammals. Babesiosis occurs more frequently and is becoming an increasing veterinary problem because changing global conditions effects the spread of ticks which serve as the vector. In Slovakia, the highest prevalence of babesiosis has been recorded in the southwestern and southeastern regions.

The aim of this study was to investigate the occurrence of babesiosis in dogs in the southwestern region of Slovakia.

MATERIALS AND METHODS

The collection of ticks from canine patients, sampling of blood and preparation of blood smears was carried out at a private ambulance in Komárno, located in southwestern Slovakia. The drag cloth (flagging) method was used to collect ticks from vegetation in this region. Blood smears were stained by the Diff Quick procedure [6] and evaluated microscopically at x×100 magnification using oil immersion. In addition, we analysed the sequence of partial 18S rRNA of babesia from ticks by means of the PCR amplification of approximately 450 bp long fragment of 18S rRNA gene using primers BJ1 (5´GTCTTGTAATTGGAATGATGG3´) and BN2 (5´TAGTTTATGGTTAGGACTACG3´) [1].

Positive samples were purified employing a commercial kit ISOLATE II PCR, supplied by Bioline, and subsequently subjected to sequencing.

RESULTS

We collected a total of 622 ticks: 464 by flagging (59 *Ixodes ricinus* and 405 *Dermacentor reticulatus*) and 158 directly from the dogs (86 *Dermacentor reticulatus* and 72 *Ixodes ricinus*). Thirty three patients with suspected babesiosis were observed in a private ambulance for the clinical signs of the disease. A loss of appetite was observed in 22 dogs (66.7 %), apathy in 19 (57.6 %) and 19 patients had fevers. Additional clinical signs included: the presence of engorged ticks on dog bodies in 21 cases (63.6 %), haematuria in 8 cases (24.2 %), anaemia in 4 cases (12.1 %), and tremor in 6 cases (18.2 %). Other non-specific signs, such as diarrhoea, vomiting, slowed down movement, ataxia and lacrimation were observed in less than 18 % of the patients. In order to confirm babesiosis, complete examination of

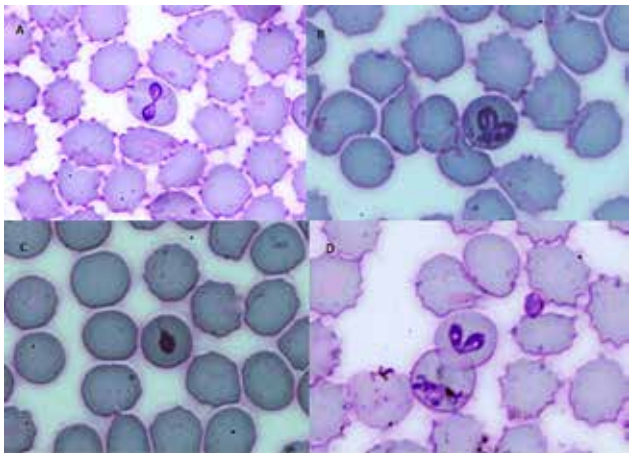

Fig. 1. Babesia in erythrocytes: A, D – Howell-Jolly bodies; A, B, D – pairs of pear-shaped babesia in erythrocytes; C – tear-shaped babesia; D – Maltese Cross-tetrahedral forms of babesia (original pictures). Magn. × 100

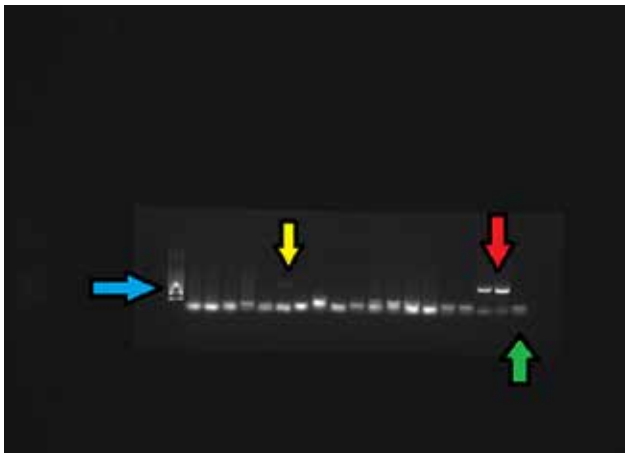

Fig. 2. Result of electrophoretic examination. Blue arrow points to DNA ladder, yellow arrow points to positive sample. Red and green arrows indicate positive and negative controls, respectively

the blood was carried out. The total number of dogs with suspicion of babesiosis was 33 and the disease was confirmed in all of them.

Blood smears from 33 patients were examined microscopically in the laboratory of the Parasitological Institute of the University of Veterinary Medicine and Pharmacy (UVMP) in Košice. The examination of blood smears showed the presence of typical forms of the parasite, such as tear-shaped forms (Fig. 1 C) and pairs of pear-shaped forms (Fig. 1 A, B, D), and also the so-called Maltese Cross-tetrahedral forms (Fig. 1 D) with light cytoplasm. The typical manifestation of haemolytic anaemia was the changed shape of erythrocytes which occurred in the form of echinocytes; the so-called Howell-Jolly bodies were also present (Fig. 1, upper left).

From the 622 collected ticks, we formed representative samples (n = 103), which were examined by the PCR for the presence of the DNA of babesia. The DNA of *Babesia* spp. was detected using this molecular method, in 2/16 ticks (prevalence 12.5 %) of the species of *Ixodes ricinus* which were collected by flagging (Fig. 2). In 1/45 ticks (prevalence 2.2 %) of *Dermacentor reticulatus* we diagnosed *Babesia canis canis*. The PCR examination of ticks collected directly from dogs presented the DNA of *Babesia* spp. in 2/21 (prevalence 9.5 %) of the *Ixodes ricinus*. The sequencing showed that one of these ticks was infected with *Babesia canis canis* (KU681325), with 490 bp sequence, which was 100 % identical with isolates of *Babesia canis* from dogs, for example from: Turkey (KF499115), Rumania (HQ662634), Croatia (FJ209025), Poland (EU622792), Russia (AY962186) and others. In 1/21 ticks (prevalence 4.8 %) of the *Dermacentor reticulatus*, we diagnosed the presence of *Babesia* spp. This was the first confirmed occurrence of DNA of *B. canis* in *Ixodes ricinus* ticks in Slovakia.

DISCUSSION

The dynamics of the spread of babesiosis in Europe has changed considerably in recent years. These changes have been ascribed frequently to the evidence of the extension of geographic territory and vertical distribution of ticks. Canine babesiosis has spread also to Slovakia, with the first clinical cases recorded in the eastern part of the country around Michalovce. Many authors in different studies have discussed the issue of the most suitable and reliable method for the diagnosis of babesia. The diagnostic methods used for the detection of *Babesia* spp. infection differ in their successfulness. *Babesia* spp. are commonly detected by microscopic examination of blood smears but this technique has some limitations with regard to its low sensitivity with low parasitaemia and difficulties with distinguishing morphologically similar species. [3]. The method based on the proof of antigens of immunofluorescent antibodies is also used to assess exposure of dogs to *Babesia* spp. infection, but the specificity of this method is low due to antigenic cross reactivity [5, 6]. This method also fails to determine whether an ongoing infection is involved. The alternative techniques, such as polymerase chain reaction (PCR), were indicated by several authors as the most suitable way of detection and identification of *Babesia* spp. [7]. Despite the low recovery of parasites at low parasitaemia, the microscopic examination of blood smears is still one of the most frequently used methods owing to its simplicity and availability [5].

The first occurrence of canine babesiosis in Slovakia was reported by Chandoga et al. in 2001 [2]. Today this disease is a common seasonal health problem in the respective regions presented. This was confirmed by the study of Kubelová et al. [4] which focused on the prevalence of *Babesia canis canis* in *D. reticulatus* ticks in southeastern, southwestern and west Slovakia. The results presented by these authors confirmed that the highest prevalence of *B. canis canis* (14.7 %) was in the area around Michalovce. A significantly lower prevalence (2.3 %) was detected in the Gabčíkovo region (southwestern Slovakia). Our results showed that in the Komárno region (southwestern) the prevalence of *Babesia* spp. in *Ixodes ricinus* ticks reached 12.5 % and that of a *Babesia canis canis* in *Dermacentor reticulatus* reached 2.2 %.

CONCLUSIONS

This study provided the first proof of the occurrence of the DNA of *B. canis canis* in ticks of the species *Ixodes ricinus*. Up to this date, no study carried out in Slovakia reported *Ixodes ricinus* as a babesiosis vector.

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THE INFLUENCE OF PROBIOTICS ON REPRODUCTIVE PARAMETERS OF SOWS AND HEALTH OF THEIR SUCKLINGS

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ABSTRACT

Thirtytwo sows were included in the trial. They were divided into the experimental group (n = 16) and a control group (n = 16). The experimental group received 1.28×10^6 *Bacillus subtilis* and *Bacillus licheniformis* per gram of feed (400 ppm BioPlus 2B, Chr. Hansen, Denmark). The trial started 2 weeks before farrowing and lasted until weaning. No significant differences were revealed in the number of piglets born alive, stillborn or the number of weaned pigs between the two groups of sows. The wean-to-first service interval was not significantly different between the groups, but sows in the experimental group had earlier first services. The conception rate did not differ. Sows in the experimental group suffered from postpartum dysgalactia syndrome (PDS) less than sows in the control group. The suckling piglets in the experimental group of sows reached better weight on day 14 of the trial and this state persisted up to the end of the experiment. The differences in the weights of the experimental group and the control group were significant at the end of the trial ($P < 0.01$). The experimental piglets had significantly lower incidence of diarrhoea than those in the control group ($P < 0.05$).

Key words: piglets; probiotics; sows

INTRODUCTION

The prosperity of sow farms depends upon the number of weaned pigs per sow per year. It is connected with other parameters, e.g. number of pigs born alive, time to the first service after weaning, the need to re-mate sows, incidence of diseases and mortality of pigs.

Farmers use various supplements to feed to gain good results. For example, probiotics have received considerable attention as a suitable growth promoter in the pig industry for many years [12]. For optimal use in a farm setting, probiotics administration should be cost-effective, stable to moisture (or portion packed) and temperature. These criteria are difficult to meet reliably for most bacteria. Nevertheless, a number of commercial preparations are available to pig farmers and have been tested relatively rigorously.

In biological terms, the easiest microbes to manipulate are those that produce spores; spores are extremely stable under normal storage conditions. Several spore-forming species of the genus *Bacillus* (*B. subtilis*, *B. licheniformis*, *B. cereus* var *toyoi*) have been used in the pig industry. Interestingly, these organisms are not usually part of the indigenous porcine gut microbiota; they are, however common soil bacteria, which are likely to be transient passengers through the guts of most outdoor reared pigs [3].

Different lactic acid bacteria, and also *Bacillus* sp., are widely used as probiotics and their use has reportedly led to health benefits against gastrointestinal disorders including diarrhoea [7]. The most critical periods in which the probiotics have been tested are the period around farrowing, the first week of life and the post-weaning period.

The treatment of sows and their litters with feed supplemented with *B. cereus* var *toyoi* reduced carriage of pathogenic *E. coli* strains and resulted in altered absolute numbers and distributions of immune cells in the piglets [10]. Piglets from the group given the microbial supplement had a reduced incidence of diarrhoea and liquid faeces; they also had higher average daily gains and feed : gain ratios [11]. Another study described a large-scale study (nearly 22 000 piglets) comparing the production characteristics when sows were fed the same diet with either a proprietary mix of *B. licheniformis* and *B. subtilis* or a standard mixture of anti-microbial growth promoters [5]. The cost of producing each kilogram of pork and all other production parameters were statistically the same showing that the probiotic supplementation was effective at replacing the non-specific chemical inhibition traditionally used in the pig industry.

A study also has been conducted to evaluate the effects of probiotic preparation, containing *Bacillus subtilis* and *Bacillus licheniformis* spores, on sows and their litters. The results of the study have shown improved sow and piglet performance [1].

The aim of our study was to observe the influence of probiotic preparation, based on *Bacillus* sp., on selected reproductive and production parameters of sows and health of sucklings.

MATERIALS AND METHODS

There were 32 sows, all with the same genetic background (Landrace, and cross-bred, Landrace x Slovak White) included into the trial. Both the control (n = 16) and the experimental group (n = 16) were balanced according to the sows parity number. Both groups consisted of 8 primiparae and 8 pluriparae.

From about 14 days before the anticipated farrowing date, the sows were housed in conventional farrowing crates. The trial lasted from 2 weeks before farrowing until weaning at 4 weeks after farrowing.

The control group was fed with the standard feed for lactating sows (OŠ-09, the farm's feed mill). The experimental group was fed with the control feed supplemented with 1.28×10^6 CFU.g^{-1} of feed (400 ppm BioPlus 2B, Chr. Hansen, Denmark). The preparation, BioPlus 2B contained equally *Bacillus subtilis* and *Bacillus licheniformis*, at the dose of 3.2×10^9 per gram of powder. In both groups the piglets received the same creep feed without additives until weaning. All piglets were given an iron preparation Ferribion (Bioveta, Czech Republic) for the prevention of anaemia on the 3rd day of life.

During the experiment, the following parameters were investigated: number born alive, number stillborn, number weaned pigs, individual weight of piglets at birth, on day 14, and at weaning. Days to first service after weaning and mated sows were also determined in the experiment. We also monitored the occurrence of diarrhoea in the suckling pigs daily. The scale for the intensity of diarrhoea was 0–3 (0 = no diarrhoea; 1 = slight; 2 = watery faeces; 3 = smelling faeces with a change of colour). The diarrhoea score for groups of piglets was calculated as follows: the diarrhoea score = sum of partial scores per pen for all days : days of monitoring

The data were used for the calculation of the average value and the standard deviation. The results between the groups were compared by the unpaired Student t-test using $P < 0.05$ as the level of significance.

RESULTS

The number of weaned piglets in the control group was higher because the number of piglets born alive was higher in that group. As only one suckling piglet died on average per every experimental and control sow, the number of born alive piglets seems to be the only reason that there was a better number of weaned pigs in the control group. The number of stillborn piglets was approximately the same in both the control and experimental groups.

No significant differences were revealed in the number of piglets born alive, stillborn and number of weaned pigs between the two groups of sows (Table 1).

Table 1. Numbers of born and weaned piglets
(mean ± SD)

Group	Live born piglets	Stillborn piglets	Weaned piglets
Experimental	9.88 ± 2.28	0.63 ± 0.81	8.88 ± 1.89
Control	10.25 ± 3.51	0.50 ± 0.73	9.56 ± 3.22

Sows in the experimental group had earlier oestrus after weaning, consequently the control group needed more time to the first service. Although there were no significant differences between the groups, as time to the first service was short in both groups, sows in the experimental group had earlier first services compared with the control group. The conception rate was approximately the same in the experimental and control groups.

The experimental piglets did not suffer as much from their diarrhoea as those in the control group ($P < 0.05$) (Table 2).

Table 2. Reproduction parameters of sows and diarrhoea score of the suckling piglets

Group	Days to first service	Conception rate	Diarrhoea score
Experimental	5.69 ± 2.18	85%	0.35 ± 0.28*
Control	5.93 ± 2.31	87%	0.62 ± 0.42*

* — $P < 0.05$

In the control group, two sows suffered with clinical signs of postpartum dysgalactia syndrome (PDS) which included loss of appetite, reddening and swelling of mammary glands after parturition and the production of little milk. In the experimental group, only one sow had mastitis (reddening and swelling of mammary gland). All sows with PDS syndrome had to be treated with antibiotic.

All litters that suffered from diarrhoea with scores of 2 or 3, were treated with antibiotics to manage the bacterial diarrhoea. All piglets which needed to be treated with antibiotics, were given tetracycline (Tetravet, Sanofi, France). Five litters from the experimental group had to be treated with antibiotics. However, antibiotics needed to be administrated in 8 litters from control group.

The suckling piglets in the experimental group reached higher weight on day 14 of the trial and this state persisted up until the end of the experiment. The differences in the weight of the experimental group and the control group were significant at the end of the trial ($P < 0.01$) (Table 3).

Table 3. Weight of suckling pigs in kg
(mean ± SD)

Group	Day 0	14th day	28th day
Experimental	1.50 ± 0.36	4.10 ± 0.85	7.46 ± 1.61*
Control	1.57 ± 0.39	3.97 ± 0.91	6.88 ± 1.67*

* — $P < 0.01$

DISCUSSION

The weighing showed that the mean weight of the experimental pigs at weaning were significantly higher than that in the control group. Our results are similar to other researchers who supplemented the diet of pigs from weaning to slaughter with *B. subtilis* and *B. licheniformis* (400 ppm BioPlus 2B). They demonstrated improvement in average daily gains (ADG) and average daily feed intake (ADFI) during both the prestarter period and the overall prestarter-finishing period [2]. On the contrary, the addition of *B. subtilis* and *B. licheniformis* (500 ppm BioPlus 2B) to the diet of finishing pigs improved the ADFI, but had no effect on ADG or the G:F ratio [8]. The variation in the results of these studies can be ascribed to several factors, e.g. the age of the pigs and the BioPlus 2B dose.

It is becoming clear that the gut microbiota of animals is critically determined at the very earliest stages after birth, the so called "microbial imprinting" [4]. Organisms that are abundant in the piglet's environment at this time have a high chance of forming a permanent association with the piglet's intestinal mucosa (true "colonisation"). It may transpire that this is the most efficient time to deliver probiotics to ensure the establishment of life-long health benefits and to produce a robust microbiota, resistant to adverse ecological shifts at times like weaning. The most efficient way to deliver probiotics to piglets may be to dose sows before and during farrowing so that she, and her environment, is satu-

rated with desirable organisms in a form whereby the piglet can acquire them as part of its natural development [3].

One of the reason why higher weights were found in the experimental group may be because of better utilisation of the feed. For example, chickens fed dried *Bacillus subtilis* var. *natto* for 28 days had significantly lower blood ammonia concentrations in the experimental group [9]. That means better utilisation of proteins and higher weight in the experimental group. The reported effects of *Bacillus* probiotics on the incidence of diarrhoea in weaned piglets were similar to ours [6].

It can be concluded that the administration of *Bacillus subtilis* and *Bacillus licheniformis* (BioPlus 2B) to sows 2 weeks before farrowing until weaning, significantly decreased diarrhoea in piglets, increased piglet weight at weaning, and shortened the time to the first service of sow after weaning.

CONCLUSIONS

The aim of this research was to document the efficacy of probiotic preparations which contained *Bacillus subtilis* and *Bacillus licheniformis* in sows. After the administration of probiotics to sows 2 weeks before farrowing until weaning it can be concluded:

1) The experimental piglets did not suffer such diarrhoea as the control piglets ($P < 0.05$). Antibiotics for the therapy of diarrhoea had to be used more often in the control group than in the experimental group.
2) The piglets in the experimental group had higher weights at the end of trial, i. e. at the time of weaning. The differences in weight between groups were significant ($P < 0.01$).
3) Sows in the experimental group suffered from PDS syndrome less than sows in the control group.
4) Sows in the experimental group had a tendency to have shorter wean-to-first service intervals compared with the control group.

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THE FIRST HAEMOLYMPH ANALYSIS OF *NHANDU CHROMATUS* TARANTULAS — BIOCHEMICAL PARAMETERS

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ABSTRACT

Tarantulas are a relatively unstudied category of invertebrate which are popular with hobbyists and increasingly used in laboratory research. As their presence in the veterinary setting is limited, very little is known about the biochemistry of their haemolymph as obtained by in house sampling and analysis. A handful of studies have been performed to attempt to establish a normal range for certain parameters in healthy members of a few particular species, but that is the extent of the current research. In this study, 12 tarantulas of the *Nhandu chromatus* species purchased as immature siblings and kept under standardised conditions for 2.5 years were anaesthetised with isoflurane and had 0.2 ml of haemolymph sampled and analysed for: total protein, glucose, calcium, phosphorous and uric acid. As well as having kept a diary of their daily feed intake and recorded dates of ecdysis, the exposure time to anaesthetic and perceived effectiveness were recorded. The variables originally proposed for this investigation are based on 2 separate feeding regimes, differing in terms of feed quantity and the effect on the aforementioned biochemical parameters. Upon receipt of the biochemical results from the first sampling, it became apparent that unexpected correlations could be made between the stage of ecdysis, susceptibility to anaesthesia and the total protein levels in the haemolymph. Those that were due to shed imminently, indicated by cessation in feeding, had recognisably and significantly higher total protein levels and reached a better level of anaesthesia in less time. Additional samplings are planned in the future to specify more definitive parameters. The observations made inadvertently so far could constitute novel information and be practically useful to tarantula enthusiasts and anaesthetists, and therefore, potentially of high clinical significance.

Key words: biochemistry; ecdysis; haemolymph; *Nhandu chromatus*; tarantula

INTRODUCTION

Little is known about the history of keeping tarantulas recreationally but the hobby anecdotally took off in the 1970s with the exportation of *Brachypelma smithi* and by the 1990s over 200 species were being bred in captivity [3]. They are currently one of the most popular non- mammalian pets, as well as being used in zoological exhibitions and as research specimens [8].

The tarantula species used in this study were *Nhandu chromatus* commonly known as the "Red and White Brazilian Bird-eater"; so named due to their relatively large size, despite probably not eating birds as part of their diet. In reality, little has been done in the way of nutritional studies of tarantulas in captivity or in the wild [8]. This species is popular in collections due to its large size and striking colouration of a red opistosoma, white prosoma and black and white striped appendages despite their highly defensive reputation [5].

The nutrients and hydration required by arachnids are usually derived from invertebrate prey species that come within reach. Once captured and immobilised, the spider will regurgitate digestive juices onto their prey and digestion begins externally.

The metabolic rate of spiders is extremely low with oxygen consumption being only 100th of that of warm blooded mammals [7]. Starvation has little effect on survival for months on end in order to enable overwintering and periods of reduced feed availability [2].

Due to the lack of a separation between the lymphatic and blood circulatory systems, the circulating fluid which carries oxygenated pigments is called haemolymph. The pigment equivalent to haemoglobin in invertebrates is copper based haemocyanin which is a clear coloured liquid that turns blue and coagulates quickly in contact with air and flows freely in the haemolymph as proteinaceous crystals [8].

Arachnids have a tubular heart which runs dorsomedially within a pericardial sinus along the anterior two-thirds of the opistosoma just ventral to the abdominal exoskeleton [11]. Haemolymph then re-enters the closed circulatory system but the venous return to the heart is via an open circulatory system free flowing amongst the tissues [4].

Within the bodies of all arachnids, a complex of prosomal and opisthosomal muscles can generate locally increased haemolymph pressure within the semi-open circulatory system. This is the mechanism by which all locomotive and tactile movements occur and can be referred to as a hydraulic system [6].

As far as we know, only 1 study has been performed analysing the biochemical parameters of tarantula haemolymph using a biochemistry machine such as those that are available to veterinarians. Eleven *Theraphosa blondi* and twelve *Grammostola rosea* tarantulas were sampled by Zachariah et al. in 2007 [12] immediately after wild capture and 8 weeks later after a period of good nutrition and hydration. Significant differences in body weight, sodium, potassium, and osmolality between the sampling times for both species were seen. There were also significant differences in: creatine kinase, calcium, total protein, and blood urea nitrogen between the sampling periods for *T. blondi*. The results of that study suggested that serial haemolymph samples may be used to assess the hydration status of theraphosid spiders. Differences seen in the biochemical results for different species in the aforementioned study, as well as those obtained by Stewart and Martin in 1970 [9] for *Dugesiella hentzi*, show the importance of defining the normal levels for each species.

The aim of this study was to determine the physiological values of selected biochemical parameters in the haemolymph of tarantulas *Nhandu chromatus* and obtain relevant information regarding their stage of ecdysis and anaesthesia susceptibility.

MATERIALS AND METHODS

The test subjects used in this study (n = 12) were purchased as immature siblings from a private breeder and subsequently kept under identical conditions for 2.5 years. They were individually housed in a quiet room with a steady temperature of 24 °C in 26 × 19 × 14 cm clear plastic boxes with perforated lids, each with a halved large yoghurt pot for shelter on a peat based substrate with high acidity to prevent fungal and parasite growth. Each box was labelled with their personal number from 1 to 12 to aid with record keeping. A small bottle top filled with water every 2–3 days was provided for ad libitum drinking, as well as additional watering of the soil to maintain a reasonable humidity when it became dry.

The vivariums were only disturbed every 2—3 days for feeding, watering and removal of mould or shed skins. Re-

cords were kept detailing the number of cockroaches fed, individuals that had shed and individuals that had not eaten since the last feeding, as well as ambient temperature and any additional notes every time they were visited.

Each individual was fed every 2 to 3 days with either 4 live, similar sized *Blatta orientalis* cockroaches or Brown crickets *Acheta domesticus*.

Anaesthesia was performed in their vivariums, with the lid from their enclosure exchanged for a non-perforated lid with a hole cut out for airtight application of the anaesthesia circuit. Each spider was anaesthetised with 5 % isoflurane (Furane, Baxter Healthcare Corporation, Deerfield, IL 60015, USA) at a flow rate of $2 l.min^{-1}$ for 5—15 minutes, depending on the effect [1].

Once removed from their vivarium, each tarantula was placed on the table and restrained with a piece of cling film held over their whole body.

An insulin syringe (BD Micro-Fine™ Plus 1ml, Becton, Dickinson and Company, 1 Becton Drive, Franklin Lakes, New Jersey, 07417-1880 201.847.6800) was used by inserting the needle through the cling film at 45° angle into the dorsal midline at the centre of the opistosoma until haemolymph could be withdrawn directly from the cardiac chamber. 0.2 ml was collected from each tarantula consecutively and transferred to Eppendorf tubes labelled with their personal number.

The cling film was then folded off the opistosoma to allow for the application of Pasco® super-glue sufficient to seal the injection site to prevent or stop bleeding.

Each individual was transferred to a bowl to be weighed and then returned to their enclosures.

The samples were then centrifuged for 5 minutes at 3.5 rpm, then a minimum of 0.1 ml was transferred to the Cobas C111 biochemical analyser (Roche Diagnostics Limited, Forrenstrasse, CH–6343, Rotkreuz, Switzerland).

The Student t-test was the test used to define statistical significance between the imminently shedding and recently shed variables for total protein, glucose and uric acid. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Every tarantula was successfully sampled and yielded biochemical data (Table 1). Results of statistical analysis for each parameter in all 12 tarantulas are presented in Table 2.

The weights of tarantulas were between 6 g and 10 g, the variety of which was to be expected as some had shed one more time than others. It was also apparent that those weighing 6 g and 7 g had shed recently which again was to be expected as they lose the weight of their previous exoskeletons and appear to have smaller abdomens after shedding.

In addition to the biochemical results, the anaesthesia times were recorded with a loose assessment of anaesthetic depth in order to obtain a better standard time for exposure to isoflurane for future samplings. During the course of the sample collection it swiftly became possible to predict each individual's response to anaesthesia by the assessment of their stage of ecdysis (Table 1). This was achieved by visual inspection of each animal and by checking their feeding and shedding records. Those that were approaching shedding had rounder abdomens with hairless patches through which new hairs could be seen growing underneath and all had stopped eating. Throughout the rearing of these test subjects, they had ceased eating for between 0 and 19 days before shedding and the time between sheds ranged from 42 to 113 days. Those that had recently shed had done so within 14 days of the sampling and one shed just 2 days before.

It was observed during the course of the anaesthesias that those fast approaching ecdysis responded to anaesthesia more swiftly and successfully than those that had recently shed, one of whom showed minimal response after 15 minutes of exposure and proved very difficult to restrain.

Upon receipt of the biochemical data, it also became visually apparent that there may be some correlation between the stage of shedding and levels of total protein, glucose and uric acid.

As is demonstrated in Table 3, only total protein showed a high statistical significance between the variables with a P value of 0.0023. It is interesting that in a majority of cases, glucose was higher in those which had not eaten for 2 weeks compared to those that had eaten 4 cockroaches within the previous 2 days.

In terms of obtaining a normal range for the species, all of the parameters seem to be without anomalies except for uric acid which shows great variations. This may be due to the temporal proximity of the last defecation, as uric acid is one of the waste products excreted along with guanine, adenine and hypoxanthine [10]. Unfortunately, speculation on the cause of this variety is beyond the scope of this study.

Table 1. The weight and biochemical results for each tarantula with time and quality of anaesthesia

Tarantula ID	Weight [g]	Total protein [g.l^{-1}]	Glucose [mmol.l^{-1}]	Calcium [mmol.l^{-1}]	Phosphorus [mmol.l^{-1}]	Uric acid [mmol.l^{-1}]	Ecdysis stage	Anaesthesia	
								Time min	Quality level
1	9	34.2	1.02	3.03	0.18	1.5	Imminent	5	Adequate
2	7	23.2	0.62	2.89	0.27	2.5	Recent	7	Adequate
3	6	26.4	0.82	3.16	0.33	0	Recent	12	Poor
4	9	25.5	0.77	3.01	0.27	2.8	28 days ago	10	Adequate
5	8	34.1	2.4	3.3	0.22	2	Imminent	10	Good
6	7	22.3	0.98	2.87	0.29	0.3	Recent	10	Poor
7	10	30.3	0.88	3.28	0.31	3.8	Imminent	8	Good
8	9	29.2	0.64	3.34	0.2	0	33 days ago	8	Poor
9	9	27.9	0.96	3.46	0.24	1,6	Recent	15	Poor
10	8	28.2	1.88	3.23	0.32	3	Imminent	10	Good
11	9	31.1	0.92	3.55	0.26	1.2	Imminent	8	Good
12	9	24	1.58	3.05	0.29	0	Just shed	12	Good

Table 2. Statistical analysis of all biochemical parameters investigated in 12 tarantulas

Parameter	Mean	Min.—Max.	Range	SD	SEM
Total protein [g.l^{-1}]	28.03	22.3—34.2	11.9	3.9585	1.427
Glucose [mmol.l^{-1}]	1.12	0.62—2.4	1.78	0.5455	1.1575
Calcium [mmol.l^{-1}]	3.18	2.87—3.55	0.68	0.2167	0.0626
Phosphorus [mmol.l^{-1}]	0.265	0.18—0.33	0.15	0.0474	0.0137
Uric acid [mmol.l^{-1}]	1.5583	0—3.8	3.8	1.3049	0.3767

SD — standard deviation; SEM — standard error of mean

Table 3. The results for total protein, glucose and uric acid divided into stages of ecdysis (excluding the 2 which were in mid-cycle) and the results of the statistical analysis by the Student *t*-test

	Total protein		Glucose		Uric acid	
	Imminent	Recent	Imminent	Recent	Imminent	Recent
	34.2	23.2	1.02	0.62	1.5	2.5
	34.1	26.4	2.4	0.82	2	0
	30.3	22.3	0.88	0.98	3.8	0.3
	28.2	27.9	1.88	0.96	3	1.6
	31.1	24.0	0.92	1.58	1.2	0
Student *t*-test	**Two-tailed** P = 0.0023*		**Two-tailed** P = 0.2506		**Two-tailed** P = 0.0759	
Mean	31.58	24.78	1.42	0.992	2.3	0.88
SD	2.574	2.324	0.6844	0.3588	1.082	1.121
SEM	1.151	1.04	0.3061	0.1604	0.484	0.501

SD — standard deviation; SEM – standard error of mean; * — P < 0.05

Fig. 1. The dorsal hyper-extension of legs seen during anaesthesia of an adult female *Grammostola rosea* in a pilot study for this experiment

All of the test subjects survived sampling with no adverse effects to either the anaesthesia or having 0.2 ml of haemolymph taken. Some individuals proceeded to shed within the next few days and they all resumed eating two days later.

Tarantulas, like other invertebrates have an exoskeleton and, must shed in order to increase in body size and reach maturity. It is well known that shedding occurs more frequently in spiders that are being fed more. "Power feeding" is used by tarantula dealers in order for their animals to reach maturity faster and fetch a higher price in less time.

As of yet there are no reports on the stage of ecdysis effecting susceptibility to anaesthesia to compare with the results gained in this study. Despite the lack of quantitative data retrieved so far, it is possible to visually compare the anaesthesia time and effectiveness in comparison to the stage of shedding.

This study is intended to continue with 3 other samples being taken a month apart to assess the effect of the feeding regimes on haemolymph biochemical parameters with the 12 tarantulas being split into two groups of 6, one fed 10 times more than the other group, then fed equal amounts the next month, then reversed. In future samplings it will hopefully be possible to accurately record the time taken before full anaesthesia is reached. This would be accomplished by implementing tests such as touching the legs or testing the self righting reflex as performed in a study by Dombrowski et al. in 2013 [1] when comparing isoflurane and carbon dioxide anaesthesia in *Grammostola rosea* tarantulas.

Another interesting observation made during anaesthesia of certain tarantulas was a strange, momentary, unnatural positioning of the limbs during induction seen in Fig. 1. It only occurred in those which responded more quickly to anaesthesia and appeared to be a hyper-extension of the legs dorsally above the prosoma, then relaxation into a normal position a few seconds later. It is possible that this previously undescribed phenomenon could act as an indicator of anaesthetic depth and will be recorded in more detail during subsequent samplings.

This interesting reflex and the observed correlation between the stage of ecdysis and anaesthesia quality, can surely contribute to the minimal knowledge currently available on tarantula anaesthesiology. Practically it may help veterinarians and researchers to predict the amount of time they should expect for full anaesthesia of tarantulas if they have access to their history. It may even prevent accidents from occurring involving more recently shed individuals who are still able to evade restraint after the same exposure to isoflurane as others.

The statistical significance in the difference between total protein in the haemolymph of tarantulas classified as recently shed and imminently shedding is also novel information. It will have to be supported by future samples and studies but may also help in the identification of the stage of ecdysis in individuals obtained without a history. In future studies, it may be possible to identify a specific level of protein that needs to be reached in the haemolymph by means of feed intake in order for healthy individuals to cease eating as is normal during pre-moult.

CONCLUSIONS

As so little is known about tarantula haemolymph, especially from *Nhandu chromatus*, any information gathered, whether to set a standard range for healthy animals or to investigate ill health, is valuable. Tarantulas are greatly under-represented in veterinary clinics, but this may change as our comprehension of their physiology improves.

Similar studies in the future will hopefully provide more and more accurate ranges for biochemical parameters and further investigate the observations made regarding the stage of ecdysis and anaesthesia susceptibility. Hopefully one day it will be possible to sample haemolymph and run biochemical analysis in veterinary clinics in order to diagnose diseases in arthropod patients with a view to treating them. This option is, as of yet, unavailable to us but this study has shown that 0.2 ml can be safely taken from 6 g tarantulas and at least 5 biochemical parameters can be obtained from this.

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A STUDY OF FISH LICE (ARGULUS SP.) INFECTION IN FRESHWATER FOOD FISH

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ABSTRACT

***Argulus* sp., commonly referred to as fish lice, are crustacean ectoparasites of fishes. The hematophagous parasites attach to and feed off the integument of their hosts. Outbreaks of epizootics have been reported worldwide, causing mass mortalities and having serious economic implications for fish farms and culture efforts. *Argulus* fish lice may also serve as vectors of infectious diseases and as intermediate hosts of other parasites. Two native European species, *A. foliaceus* and *A. coregoni*, as well as the invasive Japanese fish louse *A. japonicus,* have previously been recorded in Slovakia. This study investigated samples collected at fish farms and culture sites of Common carp (*Cyprinus carpio* L.), Pike-perch (*Sander lucioperca* L.) and Brook trout (*Salvelinus fontinalis* M.) in Eastern Slovakia, as well as samples collected from live fish imported to the Slovak Republic. A quantitative description of the of *Argulus* sp. was recorded from each locality. Samples from Common carp were identified as the invasive *A. japonicus*, and samples from Pike-perch and Brook trout were identified as *A. foliaceus*. Evidence of a mixed infection of Pike-perch with both *A. foliaceus* and *A. japonicus* was found in samples from Zemplínska Šírava, which was substantiated by electron microscopic examination. Morphometric characteristics were measured and averages and ranges produced for each species and sex.**

Key words: *Argulus foliaceus*; *Argulus japonicus*; Common carp; crustacean ectoparasites; Pike-perch; mixed parasite infection

INTRODUCTION

Fish lice is common name for branchiurid ectoparasites of fish from the genus *Argulus* [11]. Although *Argulus* fish lice are widely distributed and able to parasitize and thrive off a wide range of host species, argulosis have rarely been found to have severe effects on naturally occurring fish populations [5, 27]. Here the low intensity of infection is typically insufficient to be associated with mortality or extensive tissue damage [10]. This occurs as a result of a com-

bination of the mechanical action of the attachment organs and the feeding apparatus of the argulid, and the chemical action of enzymes or toxins injected into the host fish by the feeding fish louse [31]. Primary lesions develop at the predilection sites; haemorrhagic spots may be seen on the fins and at the base of fins, the head, and non-respiratory surfaces of the gill cavity [5]. Argulosis may lead to secondary infections, have also been linked to the transmission of infectious and other parasitic disease [6, 31].

Argulosis typically occurs on fish farms, where high stocking densities facilitate parasite transmission, while factors such as overcrowding, capture, handling and confinement may act as stressors and negatively influence the host immune response [31].

The fish lice have a direct life cycle. After mating the female will leave the host fish and deposit eggs on solid surfaces in the immediate environment [31]. Occurrence of argulosis is seasonally dependent, parasites typically reach peak abundance during summer and autumn [10].

In Europe we can only find three species of *Argulus* parasitizing freshwater fishes, namely the native *A. foliaceus* and *A. coregoni*, and the invasive and now widely spread *A. japonicus* [10, 31]. Other European species of *Argulus* have been found on marine fishes [10, 21].

This study addresses the lack of knowledge of the present situation of these parasites in economically important species of fish in Slovakia. Three species have previously been recorded in Slovakia, *A. foliaceus*, *A. coregoni* and the invasive Japanese fish louse, *A. japonicus* [9].

MATERIAL AND METHODS

Common carps (*Cyprinus carpio* L.; Cypriniformes) and pike-perches (*Sander lucioperca* L.; Perciformes) were sampled from commercial culture ponds in Eastern Slovakia, and from live fish imported from the Czech Republic and Hungary. Brook trout (*Salvelinus fontinalis* M., Salmoniformes) was sampled from a small culture pond in Košice. In the time period 2013—2015 a total of 116 Common carps, 40 Pike-perches and 2 Brook trouts were dissected.

The fishes were dissected and examined as a part of routine screening during storage or harvesting, or as a part of a diagnostic procedure on fishes displaying clinical signs of disease. A complete parasitological dissection was performed, and age category, sex, standard length and body weight was noted in the dissection protocol. The *Argulus* sp. fish lice were collected using forceps, and fixed in 4 % formalin or 70 % ethanol. Parasitological indices, such as prevalence (P; the number of hosts infected with 1 or more individuals of a particular parasite species, divided by the number of hosts examined for that parasite species), intensity of infection (ii; the number of individuals of a particular parasite species in a single infected host) and mean intensity (mi; the average intensity of a particular species of parasite among the infected members of a particular host species), and mean abundance (ma; the total number of individuals of a particular parasite species in a sample of a particular host species divided by the total number of hosts of that species examined) [1], were evaluated.

Examination of specimens was carried out using a stereomicroscope. Species determination was performed as described by previous authors [2, 22]. Morphometric mesurements were taken on the fixed specimens.

RESULTS

Species determination of *Argulus* sp.

The investigated specimens of *Argulus* sp. were identified as *A. foliaceus* and *A. japonicus*. The fish lice obtained from Common carp was identified as *A. japonicus* and from Pike-perches both *A. foliaceus* and *A. japonicus* were identified. Mixed infection with both *A. foliaceus* and *A. japonicas* was observed in Pike-perches from Zemplínska Šírava. The fish lice collected from Brook trout was identified as *A. foliaceus,* some specimens had a body length and underdeveloped second maxillae consistent with the fifth developmental stage (Table 1).

Table 1. The species of genus Argulus found infecting carp (*Cyprinus carpio* L), Pike-perch (*Sander lucioperca* L.) and Brook trout (Salvelinus fontinalis M.)

Host species	Species of Argulus	Sampled locality
Common carp	*A. japonicus*	Hrhov, Perín, Import from Czech Republic
Pike-perch	*A. foliaceus*	Paľkov-Lúčky, Zálužice-Lúčky, Bátovce
	A. foliaceus and *A. japonicus*	Zemplínska Šírava
Brook trout	*A. foliaceus*	Botanical garden in Košice

Table 2. A quantitative assessment of the parasite populations found on carp (*Cyprinus carpio* L.)

Locality	P [%]	mi	ii	Ma
Hrhov (Winter)	11.1	3	3	0.33
Hrhov (Harvest)	38.5	19.6	7.30	7.54
Perin	6.3	9	9	0.56
Import from Czechia	19.4	2	1,3	0.39
Import from Hungary	8.7	3	3	0.26

P — prevalence; ii — intensity of infection
mi — mean intensity; ma — mean abundance

Table 3. A quantitative assessment of the parasite populations found on Pike-perch (*Sander lucioperca* L.)

Locality	P [%]	mi	ii	ma
Paľkov-Lúčky	100	80.3	40—144	80.3
Zálužice-Lúčky	80	4.5	3.6	3.6
Bátovce	100	32	17—53	32
Zemplínska Šírava	100	10.2	6.14	10.2

P — prevalence; ii — intensity of infection
mi — mean intensity; ma — mean abundance

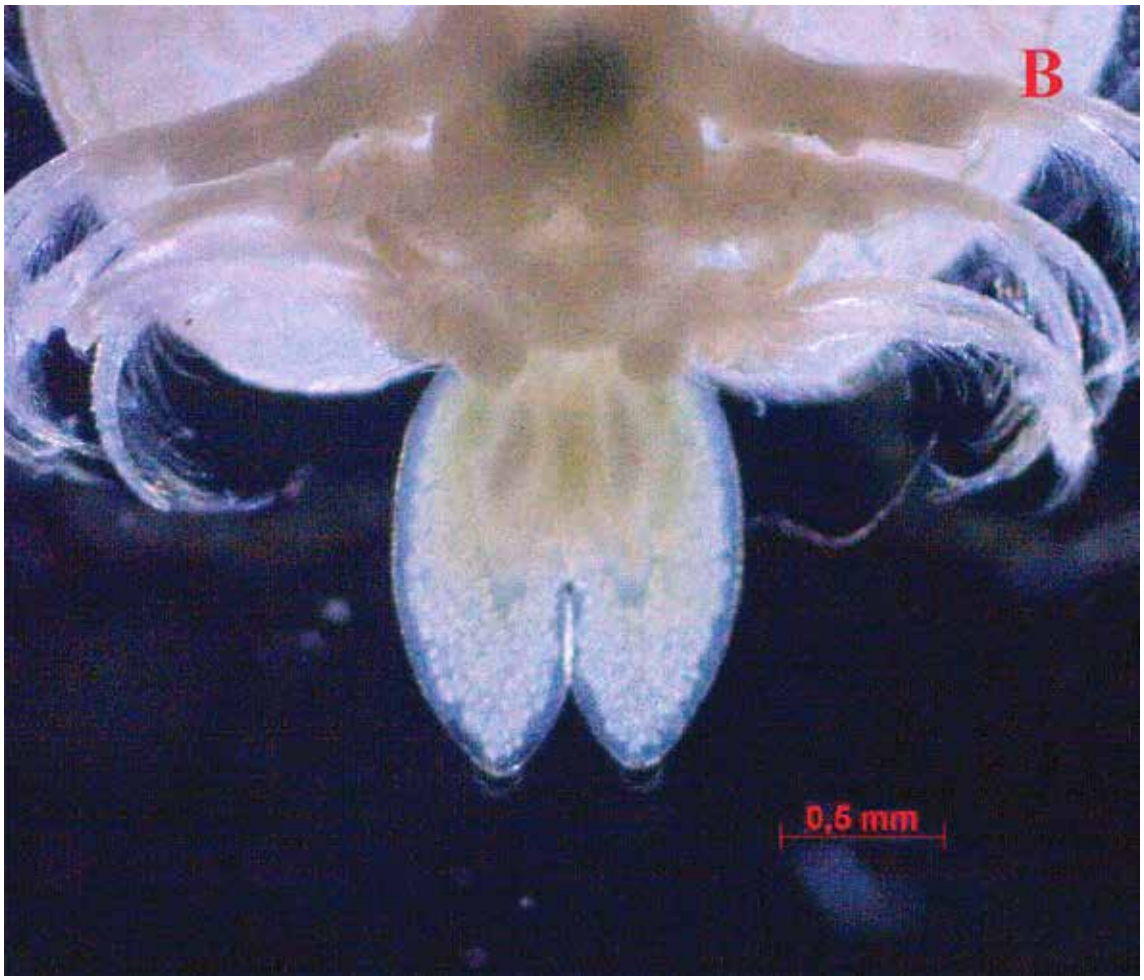

Fig. 1. Microscopic examination of *Argulus japonicus*
(A) — male on the right, female on the left, 0,8× mangnification by stereomicroscope
(B) — abdominal lobes of male. Magn. × 2.0 (stereomicroscope)

Fig. 2. Microscopic examination of males of two species: *Argulus japonicus* (left side) and *A. foliaceus* (right side)
Magn. ×0.8 (stereomicroscope)

Parasite populations found at sampled localities

The prevalence of *Argulus* infection recorded from carp varied among the sampled localities, ranging from 6.3 % to 38.5 %. The highest recorded prevalence of infection in carp was found in the pond Hrhov in the harvesting period (Table 2).

The prevalence in Pike-perch was very high, ranging from 80 % to 100 % in the sampled localities (Table 3).

Prevalence of *A. foliaceus* in Brook trout was 100 % with infection intensities ranged 25—30 and mean intensity of infection was 30 specimens per fish and mean abundance 30.

DISCUSSION

Species determination of *Argulus* sp.

The investigated specimens of *Argulus* sp. fish lice were identified as *A. foliaceus,* and *A. japonicus*.

The fish lice obtained from carp was identified as *A. japonicus*. All three European *Argulus* species have previously been recorded from carp in Slovakia [10], but only the Japanese fish louse was identified from the sampled localities. Carp infections with *A. japonicus* have been described in several studies [16, 19, 30].

In the fish lice samples obtained from Pike-perches both *A. foliaceus* and *A. japonicus* was identified. All three of the European *Argulus* species have previously been recorded from Pike-perch in Slovakia [9], but *A. coregoni* was not found among the fish lice specimens. Perchid fishes are preferred hosts of *A. foliaceus* [23], and Pike-perch infection with *A. foliaceus* have been described by previous authors [14, 32].

In three of the sampled Pike-perch localities only *A. foliaceus* was present, while a mixed infection with both *A. foliaceus* and *A. japonicas* was observed in Pike-perches from Zemplínska Šírava. Several cases of mixed *Argulus* sp. infections have been described, however the mixed infections in question involved different *Argulus* species and host fishes from our findings in Zemplínska Šírava [4, 8, 20].

Morphologically the two species *A. foliaceus* and *A. japonicus* are very similar [19, 22], and it may be impossible to determine the species of certain specimens [22]. Fish lice from Zemplínska Šírava had several characteristics consis-

tent with *A. japonicus,* however these traits are subject to variation [3]. Measurements of morphometric characteristics were found to be in range with findings of other authors [13, 18, 26, 28].

The fish lice collected from Brook trout was identified as *A. foliaceus*. Infection of Brook trout with *Argulus* sp. has not been previously recorded from Slovakia [9], however fish lice infection in this fish species have been recorded by other authors [3, 7]. *A. foliaceus* been found to infect other salmonid fish species [3, 12].

A. foliaceus from Brook trout was found to be significantly smaller than specimens obtained from Pike-perches, their body length was consistent with the sixth and seventh developmental stage [18]. Some specimens had a body length and underdeveloped second maxillae consistent with the fifth developmental stage [18].

Quantitative description of *Argulus* sp.

The prevalence of *Argulus* infection recorded from carp varied among the sampled localities, ranging from 6.3 % to 38.5 %.

The highest recorded prevalence of infection in carp was found in Hrhov in the harvesting period, greatly exceeding the prevalence from the same location during screening before and after wintering. This concurs with findings in other studies, where *Argulus* populations typically reach peak abundance during summer and autumn [6, 17].

The prevalence of infection in Pike-perch was very high, ranging from 80 % to 100 % in the sampled localities. Samples were collected in the summer and autumn, the period in which peak abundance is expected [6, 17].

The prevalence, mean intensity and abundance of *Argulus* sp. was higher in Pike-perches compared to Carp. Pike-perches are gregorious animals, where occurring in shoals may facilitate parasite transmission [29], however conflicting evidence of the effect of shoaling on *Argulus* infection exist [15]. The Zemplínska Šírava watercourse is one of the most severely PCB contaminated sites in Europe, and high levels of PCBs have been found in Pike-perches from this location previously [25]. PCBs have an immunosuppressive effect on fish and a positive correlation between PCB (polychlorinated biphenyl) pollution and abundance of ectoparasites in fish have been established [24].

The prevalence of infection in Brook trout was very high, samples were collected in the summer and autumn, the period in which peak abundance is expected [7, 26].

CONCLUSION

This study deals with *Argulus* fish lice infections of freshwater food fishes. *Argulus* fish lice are branchiurid ectoparasites capable of inflicting serious pathological effects on freshwater fishes. Little is known on the present status of fish lice in freshwater food fish in Slovakia. This study has shown that *Argulus* sp. are found parasitizing economically important species of fish in Slovakia, determined the species of fish lice are present and which species of fish they infect, and described the parasite populations found on infected fish species.

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