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Minireview

β-Glucan and parasites

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Summary

Immunosuppression caused by parasitic infections represents the foremost way by which the parasites overcome or escape the host's immune response. Glucan is a well-established natural immunomodulator with the ability to significantly improve immune system, from innate immunity to both branches of specific immunity. Our review is focused on the possible role of glucan's action in antiparasite therapies and vaccine strategies. We concluded that the established action of glucan opens a new window in treatment and protection against parasitic infections.
Keywords: glucan; parasite; Toxoplasma; Leishmania; immunity

Background

Natural products that are useful in treating various diseases have been intensively sought after throughout the history of mankind. Almost than 40 years ago, β-glucan was described as biological response modifier (BRM) that could stimulate tumor rejection in mice (Yanagawa *et al.,* 1984). As with many other BRM, it was classified as "nonspecific" because the cellular and molecular targets were unknown and its effects appeared to be highly pleiotropic and even more unpredictable. Despite long-term interest and research, the mechanism of how β-glucan affects various biological processes remained an enigma for a rather long time. Only in the last decade has extensive research by numerous scientific groups helped to reveal the extraordinary effects that β-glucan has on our immune system. A schematic representation of the basic molecular structure of β-glucan is presented in Figure 1.

For a long time, β-glucan has been studied in infections. Using several experimental models, it has been well established that β-glucan protects against infection with both bacteria and protozoa, and enhances antibiotic efficacy in infections with antibiotic-resistant bacteria. The protective effect of β-glucan was shown in experimental infections with *Candida albicans*, *Streptococcus suis*, *Plasmodium berghei*, *Staphylococcus aureus*, and *Escherichia coli*; for review, see Vetvicka and Novak (2011).

Among the well-studied pleiotropic effects of β-glucan, we can mention stimulation of both humoral and cellular immunity (Novak & Vetvicka, 2008), metabolic control of diabetes (Wursch & Pi-Sunyer, 1997), stimulation of wound healing (Browder *et al.*, 1988), stress reduction (Vetvicka & Vetvickova, 2014), attenuation of chronic fatigue syndrome (Vetvicka & Vetvickova, 2015), lowering cholesterol levels (Braaten *et al.*, 1994), and inhibition of cancer (Sima *et al.*, 2015). Readers seeking a summary of glucan actions can read a recent monography by Větvička (2013) or other additional excellent reviews (de Oliveira Silva *et al.*, 2017; Vannucci *et al.*, 2017; Bacha *et al.*, 2017; Vetvicka *et al.*, 2017; Alves da Cunha *et al.*, 2017). A schematic view on the role of glucan in stimulation of immune reactions is shown in Figure 2. For more information on innate and adaptive immunity, see Netea *et al.* (2016).

Another advantage of glucan use is that it works in many species. Glucan has been found to be active in invertebrates, including

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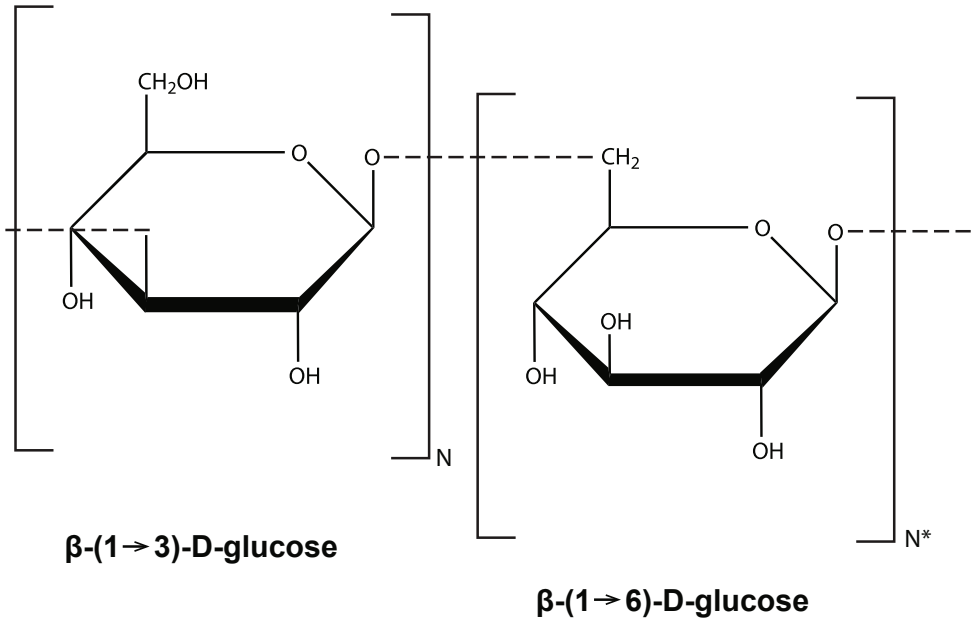

Fig. 1. Schematic representation of the basic molecular structure of glucan molecule.

earthworms (Beschin *et al.*, 1998), bees (Mazzei *et al.*, 2016), and shrimp (Duvic & Soderhall, 1990), and in vertebrates, including fish (Anderson, 1992), chickens (Vetvicka & Oliveira, 2014c), mice (Patchen & MacVittie, 1982), rats (Horvathova *et al.*, 2008), hamsters (Wang *et al.*, 1997), dogs (Vetvicka & Oliveira, 2014b), pigs (Vetvicka & Oliveira, 2014a), calves (Buddle *et al.*, 1988), and monkeys (Reynolds *et al.*, 1980), possibly making it the only immunomodulator active in every species tested. It is clear that with so many reports; several types of glucan were used, leading to the question if the same glucan will have the same results across species. So far only one study exists directly comparing effects of two different types of glucan in chicken, mice, dogs and pigs (De Oliveira *et al*, submitted). The study showed that these glucans had identical effects in all four different species. From these results, it is not surprising that glucan is intensively studied in humans, too (Kushner *et al.*, 2014; Richter *et al.*, 2014; Větvička, 2013).

Special role of glucan action has been established in invertebrates, representing one of the major defensive mechanisms. Glucan is involved in the prophenoloxidase system, and glucan-binding protein with a specific affinity to glucan plays an important role in protection of invertebrate animals, especially arthropods, against parasites and other invading pathogens; for review, see Vetvicka and Sima (2017) and Soderhall and Cerenius (1998).

Despite the fact that infections were one of the first studied actions of glucan in vertebrates, the question of glucan and parasites remains rather overlooked. At the same time, parasitic diseases are a major cause of morbidity and mortality, with more than three billion people infected worldwide (Bhutta *et al.*, 2014, Torgeson *et al.*, 2015). As most infections occur in developing countries, the need for a dependable and economical cure/prevention is particularly high. As the immune system of infected individuals seems to be particularly compromised (Samuel, 2016), an established immunostimulant, such as glucan, might be the ideal solution. The role of glucan in parasitic infection was intensively studied in the 1980s and, after three decades of neglect, the focus of glucan studies is slowly returning to this topic.

Mechanisms involved in glucan stimulation of immunological and inflammatory responses fall beyond the scope of this review. However, the most important action resulting in adequate stimulation is probably the way glucan interact with their receptors. The main glucan receptors are complement receptor 3 (CR3, CD11b/CD18) and Dectin-1. The first receptor belongs to the β_2-integrin family and is found mostly on macrophages, leukocytes, and NK cells. Glucan bind to the lectin site of this receptor and the overlapping I-domain of CD11b. The stimulation of cells relies on simultaneous binding of glucan and iC3b-opsonized material (Xia *et al.*, 1999). On the other hand, Dectin-1 is a type II transmembrane protein present on neutrophils, macrophages and dendritic cells. Upon binding of glucan, an immunoreceptor tyrosine-based activating motif is phosphorylated (Brown, 2006). In addition, stimulation of Dectin-1 receptor by glucan is mediated via Syk/NF-kB signaling axis (Fang *et al.*, 2012). For a review dedicated to the molecular interaction of glucan with receptors, see (Legentil *et al.*, 2015). The confusion regarding the effects of various route of administration was finally resolved by studies carefully comparing the effects after individual routs of administration and showing that the effects are the same (Vetvicka & Vetvickova, 2008; Vojtek *et al.,* 2017).

The development of an entirely new class of antiparasitic drugs is rare and lately seems to be near impossible. At the same time, parasitic pathogens, particularly the intracellular pathogens, are as

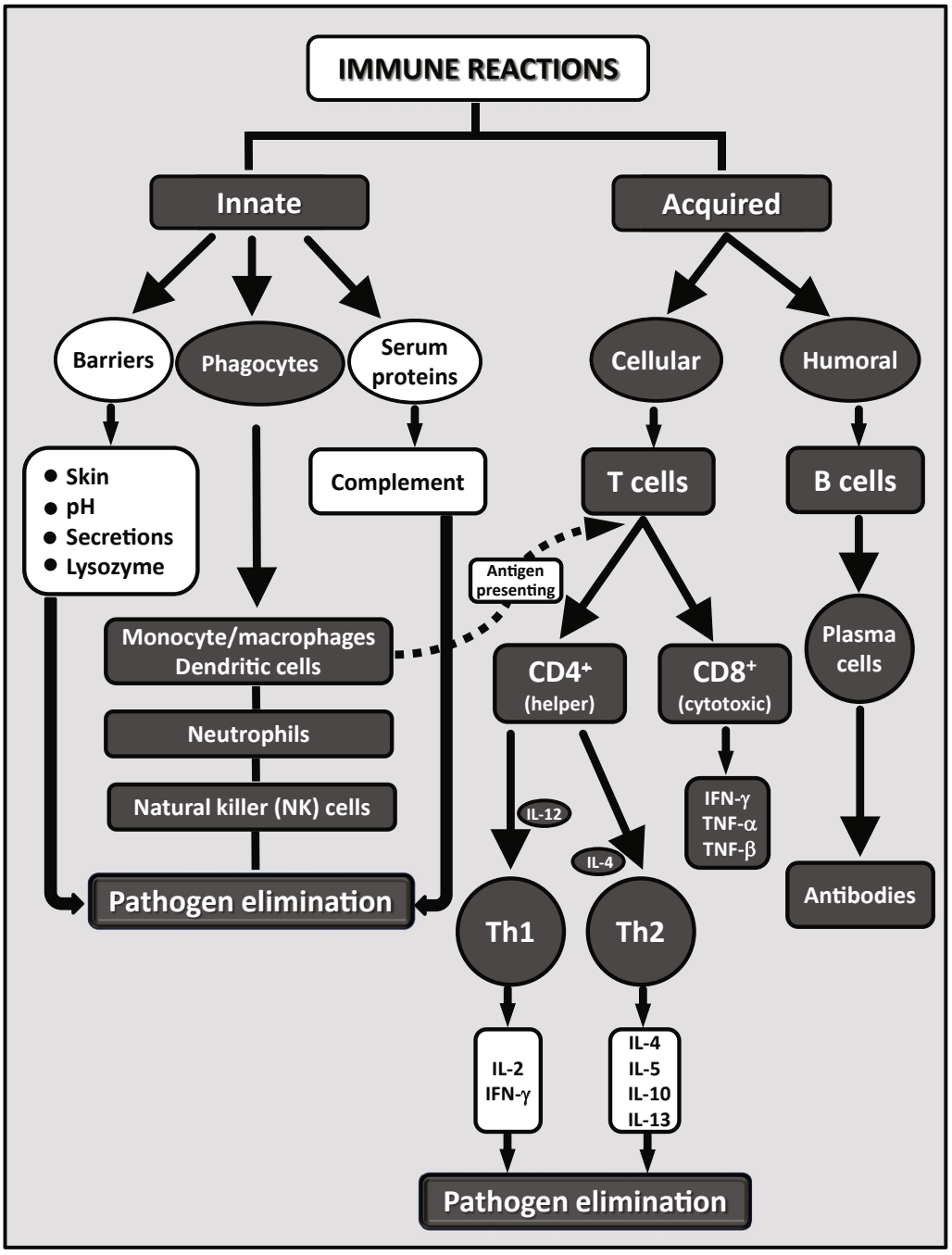

Fig. 2. Various aspects of the both branches of immune reactions. Reaction known to be influenced by glucan are represented in black, reactions where glucan has no confirmed effects are shown in white.

dangerous as ever. The question is – can glucan satisfy the need for a new drug?

Leishmania

One of the most studied parasites is *Leishmania*. Under normal conditions, the immune system cannot cope with this infection, so it is necessary to significantly boost immune reactions. An *in vivo* experiment used a genetically susceptible mouse strain infected with *Leishmania major* (Goldman & Jaffe, 1991) and showed that four intravenous injections of glucan resulted in significant suppression of infection, whereas intraperitoneal injections produced little or no effects. Older experiments showed not only protection using combination of glucan and killed *Leishmania*, but also the

positive effects of adoptive transfer by spleen cells isolated from vaccinated animals (Jarecki-Black *et al.*, 1985). Subsequent observation showed that four intraperitoneal injections prior to infection with *Leishmania* offered significant reduction in the amastigote proliferation (Al Tuwaijri *et al.*, 1987).

In an *in vitro* experimental design, infected J-774A.1 macrophages stimulated with glucan showed elevated amounts of host-protective molecules such as nitric oxide and inflammatory cytokines. Even more interesting was the synergy of glucan with a standard drug, miltefosine (Shivahare *et al.*, 2016).

Another interesting approach demonstrating the effectiveness of glucan was its use in an experimental vaccine based on *L. donovani* promastigotes. This vaccine offered significant protection regardless the route of application (Cook & Holbrook, 1983; Novak & Vetvicka, 2008). These data were based on older studies showing good effects of intravenous glucan injections and even stronger stimulation by glucan-promatostigotes combination (Cook & Holbrook, 1983). Similar data were obtained using a hamster model, where besides the effects of a glucan-prostigmatoses combination, significant protection was found after application of glucan alone, both *in vivo* and *in vitro* (Cook *et al.*, 1982). The resistance caused by injection of glucan lasted up to 80 days (Holbrook *et al.*, 1981b). Glucan alone offered protective effects in combination with every antigen fraction tested (Obaid *et al.*, 1989). Similar results were obtained on a model of *L. infantum* (Lasarow *et al.*, 1992). Glucan offered protection against *L. amazonensis* via stimulation of NK cell activities (Yatawara *et al.*, 2009). It is important to note that whereas different authors used different routes of glucan administration, the effects of glucan were same application (Cook & Holbrook, 1983; Novak & Vetvicka, 2008), which further supports the fact that glucan is active via all ways of administration.

Another study found that a 45-day application of glucan eliminated the spleen and liver parasite burden in a model of visceral leishmaniasis. Detailed analysis suggested the importance of glucan-mediated production of interleukin-12 and interleukin-17 (Ghosh *et al.*, 2013).

Other infections

A similar study evaluating the synergy between antihelmintic drug praziquantel and glucan used mice infected with *Mesocestoids vogae* tetrathyridia. The results showed that combined treatment resulted in suppression of fibrogenesis in the liver cell protection against oxidative damage, and possible stimulation of parenchyma regeneration (Velebny *et al.*, 2008). The same group previously reported that a synergistic therapy with glucan and praziquantel increased macrophage activity and resulted in increased immunoglobulin levels to the secretory antigens, but decrease to the somatic antigens, probably caused by changes in antigen exposure (Hrckova *et al.*, 2007).

Toxoplasma gondii is a common intracellular parasite, particularly dangerous for immunocompromised individuals, as the infection results in suppression of cell-mediated branch of immune reactions. The use of glucan stimulated the production of interleukin-10 in infected animals more than the standard drug, sulfadiazine (Picka *et al.*, 2005), but the relevance to the potential treatment is unclear, as the study did not measure the possible changes in parasitic load.

Older studies showed 100 % protection against formalin-killed erythrocytic stages of *Plasmodium berghei* after simultaneous intravenous glucan injections (Holbrook *et al.*, 1981a). Mushroom-derived lentinan was used during blood-stage infection with *Plasmodium yoelii*. When used as a prophylaxis, glucan strongly decreased parasitemia and increased overall survival rate. Stimulation of Th1 (subset of T helper lymphocytes) response was suggested due to the stimulation of nitric oxide, interleukin-12, and interferon-γ production. In addition, this study found stimulation of dendritic cell maturation and reduced Treg (regulatory T lymphocytes) action (Zhou *et al.*, 2009). As this group used lentinan, glucan already approved for clinical use, the results have strong clinical potential.

In a study of *Eimeria vermiformis* infection, mice were first immunosuppressed with dexamethasone, then infected with oocysts of *E. vermiformis*, and finally treated with oat β-glucan by intragastric or subcutaneous routes (Yun *et al.*, 1997). Fecal oocyst shedding was reduced in the glucan-treated groups compared to the control group. Immunosuppressed mice which received no glucan treatment showed more severe clinical signs of the disease and a 50 % mortality, while minimal clinical signs and no mortality were recorded in the glucan-treated groups. In addition, all classes of immunoglobulin showed elevated levels. Yun *et al.* (2003) later showed that oat glucan lowered fecal oocyst shedding by 40 %, probably via changes of lymphocyte populations in various lymphatic organs.

In aquaculture, glucan represents an important part of the supplements-driven stimulation of immune system. As infection with parasitic ciliate *Ichthyiohthirius multifiliis* is often fatal, it is not surprising that glucan-supplemented food was tested as possible protection. Comparing short- and long-term applications, the study confirmed the need for longer administration of glucan (Lauridsen & Buchmann, 2010). A similar study showed significant protection after longer application (Jaafar *et al.*, 2011). In addition, glucan offered protection against *Loma salmonae* given either intraperitoneally or orally (Guselle *et al.*, 2010), offering a new window for successful vaccination of commercially farmed fish. Later studies using common carp, however, did not confirm these results (Herczeg *et al.*, 2017). For a summary on glucan-derived stimulation of immune reaction in case of *L. salmonae* infection, see Rodriguez-Tovar *et al.* (2011).

Glucan combined with zinc and porcine immunoglobulins significantly reduced the number of larvae in *Toxocara canis* infections (Soltys *et al.*, 1996). As the authors never explained the reasons behind this particular combination, the results are difficult to interpret. Later experiments showed that glucan alone, when applied

with a highly infective dose of *T. canis* eggs, showed strong stimulative and restoration effects (Boroskova *et al.*, 1998).

Other aspects

Besides stimulating antiparasitic immunity and subsequently suppressing the parasitic infection, glucan can also be involved in a completely different role. In addition to helping to protect the host, glucan can also play a direct role in life of the parasite. Glucan is present in oocyst walls of *Toxoplasma* and *Eimeria*, where its fibers are part of trabecular scaffold in the inner layer of oocyst wall, but it is not a component of sporocyst and tissue cyst walls. This glucan might be targeted by drugs specific for glucan synthase (Bushkin *et al.*, 2012). However, the absence of glucan in tissue cysts suggests that glucan receptors are not involved in human innate and acquired immune responses to *Toxoplasma*.
In the case of the intracellular pathogen *Histoplasma capsulatum*, the binding of the pathogen to the membrane of macrophages is mediated by the glucan present in the cell walls. In addition, alpha glucan is important for *H. capsulatum* virulence; whereas, glucan is antigenic and are involved in modulation of the host immune response (Gorocica *et al.*, 2009). Unfortunately, no additional information is currently available.

Conclusion

In two waves of scientific interest, spanning three decades, glucan studies have consistently shown its ability to offer solid protection against parasitic infections. Despite positive effects, glucan treatment has, in general, been considered questionable, particularly due to the problems with obtaining the same glucan in subsequent batches (which is inherited problem to most natural molecules), and to the lack of knowledge of the mechanisms of action. Some of the confusing results originally reported might be contributed to the lack of high-quality glucan available at the time of those studies.
The overwhelming conclusion reached from this review is that, as an adjuvant, glucan can be as effective as, and at the same time safer than, conventional bacterial or other adjuvants (Roohvand *et al.*, 2017; Li & Wang, 2015; De Smet *et al.*, 2014). In the last decade, our knowledge of glucan and its mechanisms of action have improved tremendously. In addition, large companies are now able to produce large batches of glucan, allowing the researchers to work with identical glucan for many years. Our short review described the current knowledge of glucan action in various parasitic infections. We believe that glucan application might open a new window in treatment and protection against parasitic infections via development of vaccines.

Conflict of interest

Authors declare no conflict of interest.

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Serotonin and neuropeptide FMRFamide in the attachment organs of trematodes

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Summary

The serotoninergic and FMRFamidergic nervous system of the attachment organs of trematodes were examined using immunocytochemical techniques and confocal scanning laser microscopy. Adult trematodes from eight families as well as cercariae and metacercariae from ten families were studied. TRITC-conjugated phalloidin was used to stain the muscle fibres. The serotonin- and FMRFamide-immunoreactive (IR) nerve cells and fibres were revealed to be near the muscle fibres of the oral and ventral suckers of the trematodes and their larvae. The results indicate the important role of neurotransmitters, serotonin and neuropeptide FMRFamide in the regulation of muscle activity in the attachment organs of trematodes and can be considered in perspective for the development of new anthelmintic drugs, which can interrupt the function of the attachment organs of the parasites.
Keywords: Trematodes; attachment organs; neurotransmitters; serotonin; FMRFamide; nervous system

Introduction

The parasitic flatworms, trematodes have well-developed adhesive organs represented by oral and ventral suckers. These allow parasites to adhere to the substrate (i.e., to the host organs and tissues) and play an important role in their feeding and, in some cases, their locomotion. The musculature of its adhesive organs consists of longitudinal, circular and radial muscle fibres. Contractions of longitudinal (meridionally oriented) muscles open the sucker while contractions of circularly arranged fibres, in conjunction with the radial muscles that run between the inner and outer surfaces of the sucker close it (Mair *et al*., 1998; Halton & Maule, 2004; Yastrebov & Yastrebova, 2014; Krupenko & Dobrovolskij, 2015).

It is well known that diseases caused by parasitic flatworms are of medical and agricultural problem as they are harmful to human health as well as they are producing great economic losses in agriculture. That is why studying this animal group is important, not only for solving fundamental medical problems, but also for having great practical significance.

The nervous system of trematodes is well differentiated, consisting of central and peripheral parts, which participate in the regulation of many functions including feeding, locomotion, reproduction and migration. Data on the innervations of trematodes' fixation organs are limited but allowing us to assume on an important role of nervous system in the mechanisms of adhesion of parasites to the host tissues (McKay *et al*., 1990, 1991; Niewiadomska *et al*., 1996a,b; McVeigh *et al*., 2009; Leksanboon *et al*., 2012).

Several neuronal signal substances, including serotonin and FMRFamide-related peptides (FaRPs) have been identified in central and peripheral nervous systems of flatworm parasites indicating the neurochemical complexity of their nervous system (Gustafsson 1987; Magee *et al*., 1993; Terenina *et al*., 2006). Serotonin (5-hydroxytryptamine, 5-HT) appears to be the dominant biogenic

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amine in Platyhelminthes. In trematodes 5-HT was identified by number of biochemical methods in crude extracts of *Schistosoma haematobium*, *S. japonicum*, *S. mansoni* (Bennett *et al*., 1969; Chou *et al*., 1972), *Haplometra cylindracea*, *Opisthorchis felineus*, *Azygia lucii*, *Codonocephalus urnigerus* (Terenina & Gustafsson, 2003). Immunocytochemical studies have verified the presence of serotonin in all trematode species examined so far. 5-HT immunoreactivity has been demonstrated in their central and peripheral nervous systems: in cerebral ganglia, lateral nerve cords, transversal commissures, in subepithelial and submuscular nerve plexuses.

The FMRFamide is a member of the neuropeptides FaRPs family and was firstly isolated from the mollusks *Macrocalista nimbosa* (Price & Greenberg, 1977). So far the only four authentic FaRPs have been isolated from flatworms: YIRFamide (from turbellarian *Bdelloura candida)*; GYIRFamide (from turbellarian *B. candida*, *Dugesia tigrina*); RYIRFamide (from turbellarian *Artioposthia triangulate*), GNFFRFamide (from cestode *Moniesia expansa*) (see McVeight *et al*, 2009 for references). None of FaRPs was isolated from trematodes, but numerous immunocytochemical investigations using different antibodies (such as anti-RF, anti-GYIRF and anti-FMRF) indicate that FaRPs are broadly expressed in their nervous system (Gustafsson *et al*., 2002). Endogenous FMRF-like peptides are remarkably potent in parasitic worms (Day & Maule, 1999) and there are reasons to believe that peptidergic signalling could be an attractive target for new anthelmintic drugs developing to treat the infections (Mousley *et al*., 2005). In this context, the attachment organs of parasites may be a convenient model for drug investigations which will interfere with the function of suckers and thus entire parasite's adhesion to the host tissues.
Current research is focusing on the innervations of the musculature of the oral and ventral suckers of trematodes and its larvae (cercariae and metacercariae) with serotoninergic and peptidergic (FMRFamidergic) structures. The results obtained in this study allowed to extend our knowledge about nervous system of attachment organs in selected trematode species, their potential functions and could stimulate further research in this field.

Materials and Methods

Trematodes and fixation procedure
Specimens of adult trematodes (from eight families) and larvae (cercariae and metacercariae) from ten families, collected in various regions in Russia and Belarus were used in the study (see Table 1).
The material was fixed in 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (PBS, Sigma) at 4 °C and at a pH of 7.4. For storage, it was transferred to the PBS buffer with 10 % sucrose. Part of the samples (adult trematodes) was embedded in Tissue-Tek, frozen and sectioned at 20µm on a Bright cryostat. The sections were collected on chrom-alum-gelatine-coated glass slides, dried for approximately two hours at room temperature, and were either stained directly or stored at -20 °C. Other samples (cercariae and metacercariae) were stained as whole mounts.

Table 1. The investigated species of trematodes.

Adults
Opisthorchis felineus Rivolta, 1884 (Opisthorchiidae Loos, 1899)
Allocreadium isoporum Looss, 1984 (Allocreadiidae Looss, 1902)
Plagiorchis laricola Skrjabin, 1924 (Plagiorchiidae Lühe, 1901)
Opisthioglyphe ranae Frölich, 1791 (Plagiorchiidae Lühe, 1901)
Gorgodera cygnoides Zeder, 1800 (Gorgoderidae Looss, 1899**)**
G. loossi Sinitzin, 1905 (Gorgoderidae Looss, 1899**)**
Paramphistomum cervi Zeder, 1790 (Paramphistomidae Fischoeder, 1901)
Aspidogaster conchicola K.Baer, 1827 (Aspidogastridae Poche, 1907)
Cercariae
Cercaria parvicaudata Stunkard and Shaw, 1931 (Renicolidae Dollfus, 1939)
Plagiorchis elegans Rudolphi, 1802 (Plagiorchiidae Lühe, 1901)
Cryptocotyle lingua Creplin, 1825 (Heterophyidae Leiper, 1914)
Trichobilharzia szidati Neuhaus, 1952 (Schistosomatidae Stiles and Hassall, 1898)
Bilharziella polonica Kowalewski, 1895 (Schistosomatidae Stiles and Hassall, 1898)
Sphaerostomum globiporum Rudolphi, 1802 (Opecoelidae Ozaki, 1925)
Moliniella anceps Molin, 1859 (Echinostomatidae Looss, 1899)
Himasthla elongata Mehlis, 1831 (Echinostomatidae Looss, 1899)
Metacercariae
Leucochloridiomorpha lutea von Baer, 1826 (Leucochloridiomorphidae Yamaguti, 1958)
Cotylurus sp. (Strigeidae Railliet, 1919)

Immunocytochemistry
Whole mounts and cryostat sections of worms were stained with rabbit anti-5-HT (Instar, USA) (1:500) or rabbit anti-FMRFamide (Peninsula, Belmont, CA, USA) (1:500) primary antibodies in PBS containing 1 % (v/v) Triton X 100 (Sigma) (PBS-T) according to the method described by Coons *et al*. (1955). The whole mounts (cercariae and metacercariae) were incubated with the primary antibody for five days at 4 °C, and with the secondary goat anti-rabbit Alexa 488 (Molecular Probes, USA) (1:400) antibodies in PBS-T over five days at 4 °C. The sections were incubated with the primary antibody for two days and with the secondary antibody for three hours. Controls included omission of the primary antibody and the substitution of the primary antibody with non-immune rabbit serum.

Table 2. Serotonin and neuropeptide FMRFamide in the nervous system of the attachment organs of trematodes.

Species	5-HT	FMRFamide	References
Adults			
Bucephaloides gracilescens	+	+	Stewart *et al*., 2003a
Haplometra cylindracea	+	+	McKay *et al*., 1990
Fasciola hepatica	+	-	Fhairweather *et al*., 1987
- « -	-	+	Magee *et al*., 1989
Schistosoma mansoni	+	+	Mair *et al*., 2000
Opisthorchis felineus	+	+	Tolstenkov *et al*., 2010
Opisthorchis felineus	+	+	our data
Allocreadium isoporum	+	-	- « -
Plagiorchis laricola	-	+	- « -
Gorgodera cygnoides	+	-	- « -
G. loossi	+	-	- « -
Paramphistomum cervi	+	-	- « -
Aspidogaster conchicola	+	-	- « -
Cercariae			
Echinostoma caproni	+	+	Šebelova *et al*., 2004
Cercaria emasculans	+	-	Pan *et al*., 1994
Neoastiotrema trituri	+	+	Tolstenkov *et al*., 2012a,b
Cathaemasia hians	+	-	- « -
Echinostoma revolutum	+	-	- « -
Paramphistomum cervi	+	-	- « -
Psilohasmus oxyurus	-	+	- « -
Opisthorchis felineus	-	+	Tolstenkov *et al*., 2010
Moliniella anceps	+	-	our data, Tolstenkov *et al*., 2012a
Bilharziella polonica	+	-	- « -
Trichobilharzia szidati	+	-	- « -
Plagiorchis elegans	-	+	our data
Himasthla elongata	+	+	- « -
Cryptocotyle lingua	+	+	- « -
Cercaria parvicaudata	+	+	our data, Tolstenkov *et al*., 2011
Neoastiotrema trituri	+	+	Tolstenkov *et al*., 2012a,b
Cathaemasia hians	+	-	- « -
Echinostoma revolutum	+	-	- « -
Paramphistomum cervi	+	-	- « -
Psilohasmus oxyurus	-	+	- « -
Metacercariae			
Diplostomum sp.	+	+	Barton *et al*., 1993
Cotylurus erraticus	+	+	- « -
Opisthorchis viverini	-	+	Lecsanboon *et al*., 2012
Bucephaloides gracilesctns	+	+	Stewart *et al*., 2003a
Apatemon cobitidis proterorhini	+	+	Stewart *et al*., 2003b
Leucochloridiomorpha lutea	+	+	our data
Cotylurus sp.	+	-	- « -

+ substance detected; - substance not detected

Staining of musculature with TRITC-conjugated phalloidin

In order to study the relationship between the patterns of the FMRFamide-IR and 5-HT-IR nervous elements and the musculature, staining with TRITC-conjugated phalloidin (Sigma, USA) (1:200) was performed according to the method described by Wahlberg (1998).

Confocal scanning laser microscopy

The specimens stained with anti-5HT, anti-FMRFamide and TRITC-labelled phalloidin were examined using a fluorescent microscope Leica DM 1000 (A.N. Severtsov Institute of Ecology and Evolution of Russian Academy of Sciences, Center of Parasitology), a Leica TCS SP5 confocal scanning laser microscope (The Pushchino Scientific Center of Russian Academy of Sciences) and

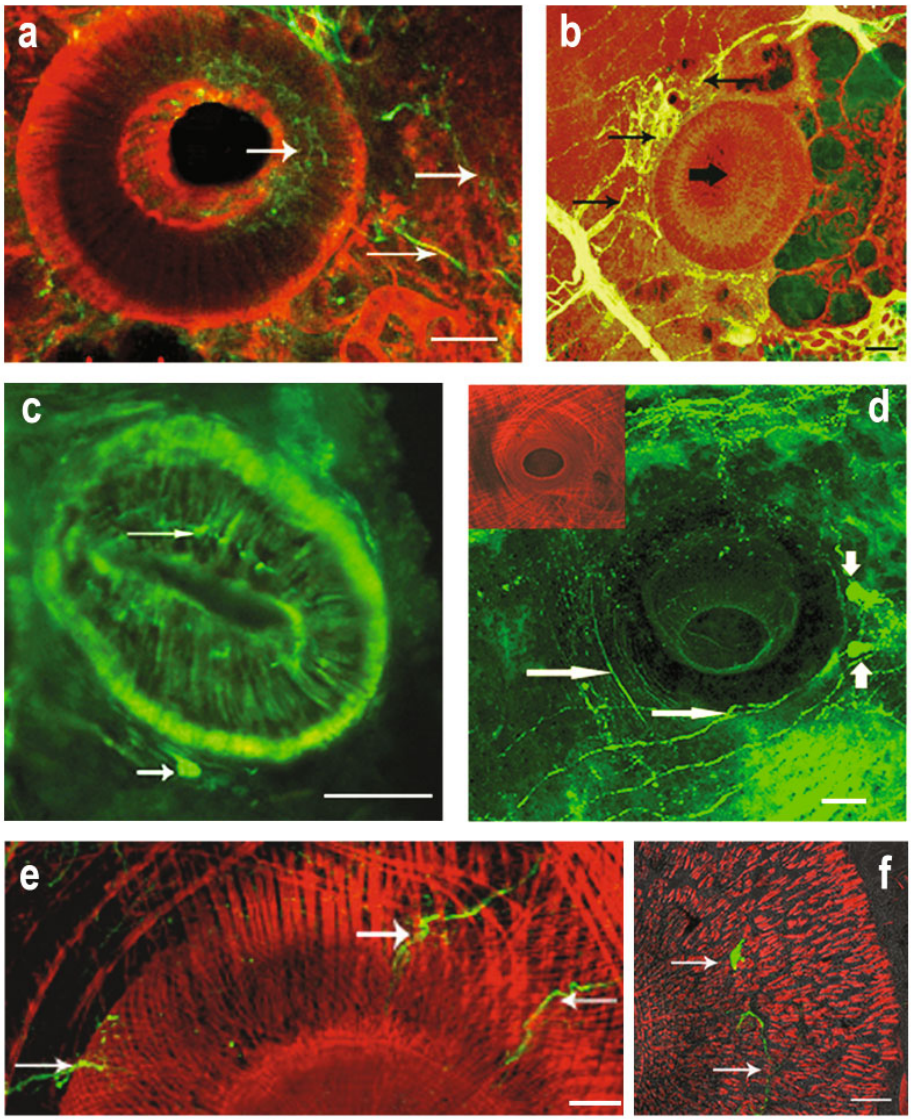

Fig. 1(a – f). The serotoninergic and FMRFamidergic components of the nervous system in the attachment organs of adult trematodes.
(a, b) ***Opisthorchis felineus*:** (a) 5-HT-immunoreactive (IR) nerve cells and fibres located near and inside the ventral sucker (arrows), scale bar 50μm;
(b) FMRFamide-IR fibres (thin arrows) extending from the main nerve cords towards the ventral sucker (thick arrow) are indicated, scale bar 50μm;
(c) ***Allocreadium isoporum***. 5-HT-(IR) nerve cell and fibres located near and inside of the oral sucker (arrows), scale bar 100μm;
(d) ***Plagiorchis laricola.*** FMRFamide-IR nerve cells (short arrows) and fibres (long arrows) located close to the ventral sucker, scale bar 30μm.
Inset: the pattern of TRITC-phalloidin labeled F-actin in the ventral sucker;
(e) ***Gorgodera cygnoides.*** Max projection shows the pattern of 5-HT-IR nerve fibres (arrows) among the muscle fibres of the ventral sucker staining with TRITC-phalloidin, scale bar 50μm;
(f) ***Paramphistomum cervi.*** 5-HT-IR nerve fibres located among the muscles of the ventral sucker (arrows), scale bar 100μm.

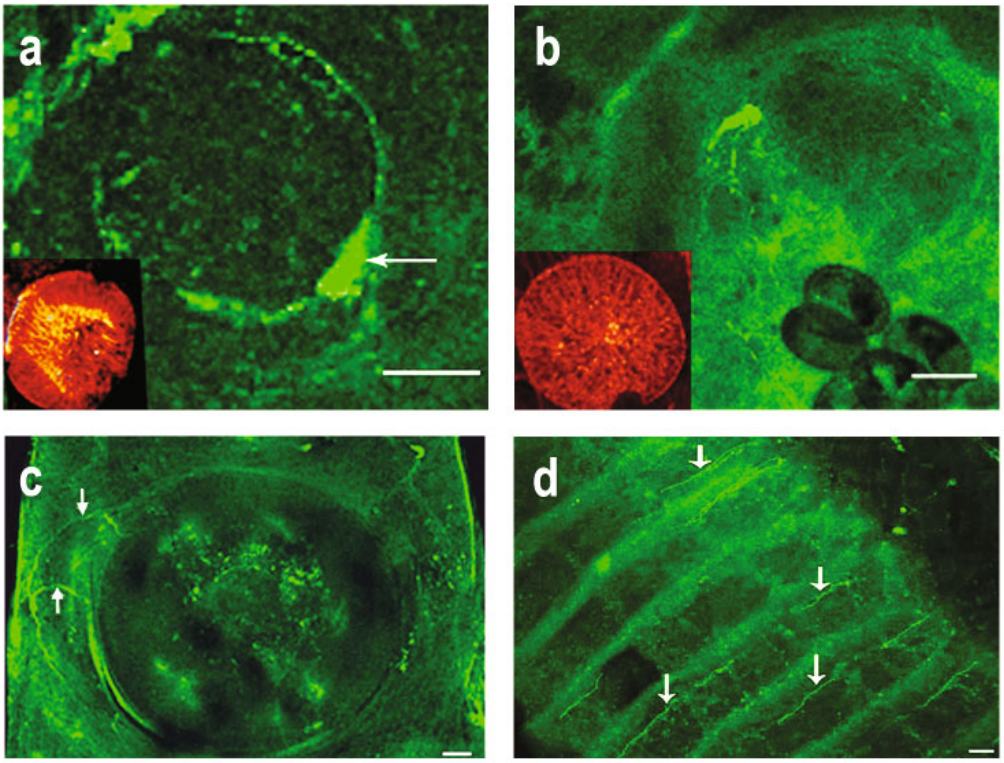

Fig. 2(a – d). The serotoninergic and FMRFamidergic nerve elements in the attachment organs of adult trematodes.
(a, b) ***Opisthiogliphe ranae.*** 5-HT-IR nerve cells and fibres located near the oral (a) (large arrow) and ventral (b) suckers, scale bar 30µm.
Inset: the pattern of TRITC-phalloidin labeled F-actin in the suckers.
(c) ***Gorgodera loossi.*** 5-HT-IR nerve cell located near the ventral sucker.
Note the 5-HT-IR nerve fibres extending to the musculature of the ventral sucker (arrows), scale bar 50µm;
(d) ***Aspidogaster conchicola.*** 5-HT-IR nerve fibres observed in the adhesive disc situated on the ventral body surface (arrows), scale bar 20µm.

a Leica TCS 4D confocal scanning laser microscope coupled to a LeitzAristoplan fluorescence microscope (The Department of Biology, Abo Akademi, Finland).

Ethical Approval and/or Informed Consent

All applicable institutional, national and international guidelines for the care and use of animals were followed.

Results

Adults

Serotonin (5-HT)- and FMRFamide-immunoreactive (IR) nerve cells and fibres were found among the muscle fibres of the oral and the ventral suckers of the adult trematodes. The positive 5-HT-IR and FMRFamide-IR was also discovered in cells and nerve fibres located near the attachment organs. 5-HT- and FMRFamide-IR fibres extending from the main nerve cord towards the ventral sucker were found in *Opisthorchis felineus* (Fig. 1a, b). Inside the ventral sucker, a network of 5-HT-IR nerve fibres can be seen (Fig. 1a). 5-HT- and FMRFamide-IR nerve cells and fibres were also found in the oral sucker of *Allocreadium isoporum* (Fig. 1c) and in the ventral sucker of *Plagiorchis laricola* (Fig. 1d), *Gorgodera cygnoides* (Fig. 1e) and *Paramphistomum cervi* (Fig. 1f). 5-HT-IR nerve cells and fibres can be seen near the muscles of the oral (Fig. 2a) and ventral (Fig. 2b) suckers of *Opisthiogliphe ranae*. Thin, 5-HT-IR fibres innervate the ventral sucker of *Gorgodera loossi* (Fig. 2c). The adhesive disc located on the ventral surface of *Aspidogaster conchicola*, a representative of the ancient trematode group, is strongly innervated by 5-HT-IR nerve fibres (Fig. 2d).

Cercariae

The 5HT- and FMRFamide-ergic nerve structures have been found in attachment organs of trematode larvae – cercariae and metacercariae. Figs. 3 and 4 show the FMRFamide-IR fibres running to the ventral and oral suckers of *Cercaria parvicaudata* (Fig. 3a, b) and *Plagiorchis elegans* (Fig. 3c, d), the ventral sucker of *Cryptocotyle lingua* (Fig. 3e) and the oral sucker of *Moliniella anceps* (Fig. 4e) and *Himasthla elongata* (Fig. 4h).

Positive 5-HT-immunoreactivity has been revealed in the nerve fibres running to the ventral sucker of cercariae of *Cryptocotyle lingua* (Fig. 3f), the oral and ventral suckers of *Trichobilharzia szi-*

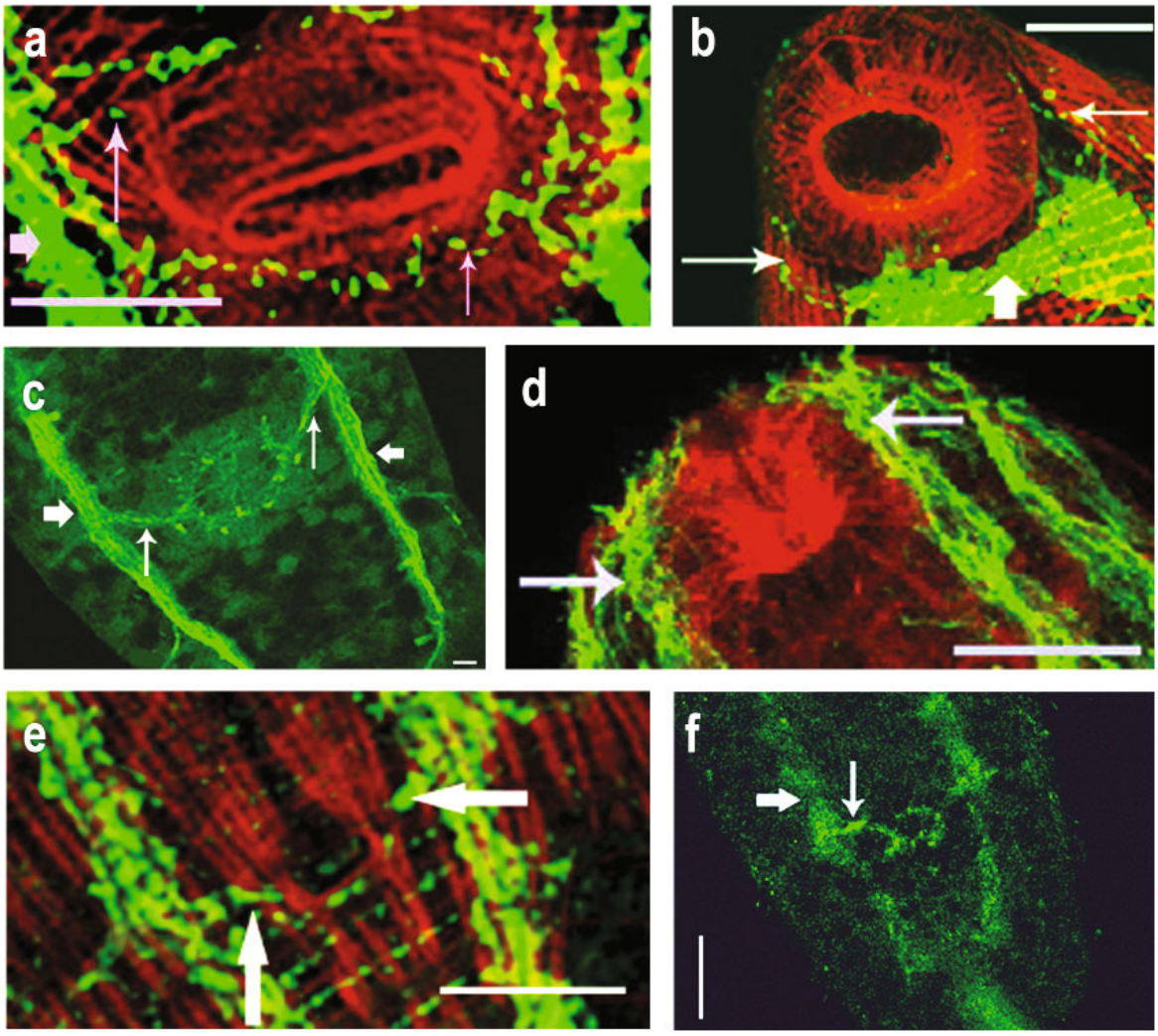

Fig. 3(a – f). The serotoninergic and FMRFamidergic components of the nervous system in the attachment organs of cercariae.
(a, b) ***Cercaria parvicaudata***. FMRFamide-IR nerve fibres extended to the ventral (a) and oral (b) suckers (arrows).
The brain commissure is marked with a thick arrow (b). Scale bar 20μm;
(c, d) ***Plagiorchis elegans.*** FMRFamide-IR nerve fibres located near the ventral (c) and oral (d) suckers (long arrows).
The main nerve cords are marked with a thick arrows (c), scale bar 20μm;
(e, f) ***Cryptocotyle lingua.*** FMRFamide-IR(e) and 5-HT-IR(f) fibres near the ventral sucker (long arrows).
The main nerve cord is marked with a thick arrow (f), scale bar on (e) - 15μm; on (f) -20μm.

dati (Fig. 4a, b), the ventral sucker of *Bilharziella polonica* (Fig. 4c), *Sphaerostomum globiporum* (Fig. 4d) and *H. elongata* (Fig. 4g), and the oral sucker of *M. anceps* (Fig. 4f).

Metacercariae

The ventral sucker of *Leucochloridiomorpha lutea* metacercariae is strongly innervated with 5-HT-IR and FMRFamide-IR nervous fibres extending from the main longitudinal nerve cords (Fig. 5a, b). The innervations of the ventral sucker with 5-HTergic fibres have been observed in the metacercariae of *Cotylurus sp.* (Fig. 5c). The summary data relating to the identification of 5-HT- and FMRFamide-IR components of the nervous systems in the oral and ventral suckers of trematodes are presented in Table 2.

Discussion

Due to well-developed muscle elements of the oral and ventral suckers, trematodes are able to attach themselves securely to the host organs and tissues. The innervations of the attachment organs are not always mentioned in the description of a general morphology of the trematodes nervous system . Only a few studies pointed out the presence of nerve structures in trematodes' oral and ventral suckers. Serotoninergic and peptidergic nerve fibres have been found in the oral suckers of the adults of *Bucephaloides gracilescens* and in the oral and ventral suckers of *Haplometra cylindracea*, *Schistosoma mansoni*, *Fasciola hepatica* and *Opisthorchis felineus* (Stewart *et al*., 2003a; McKay *et al*., 1990,

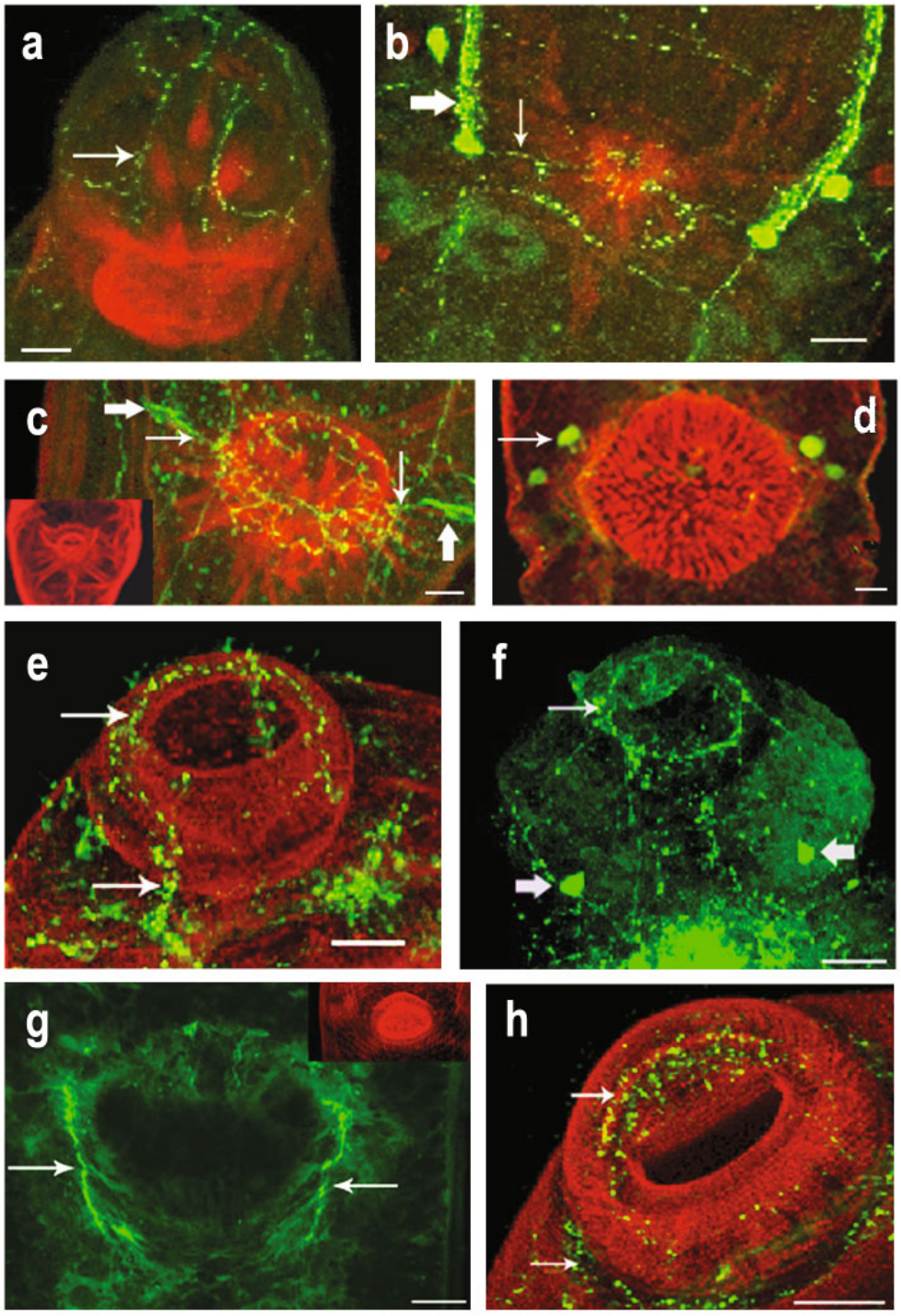

Fig. 4(a – h). The serotoninergic and FMRFamidergic components in the attachment organs of cercariae of trematodes.
(a, b) ***Trichobilharzia szidati.*** 5-HT-IR fibres in the oral (a) and ventral (b) suckers (arrows); The main nerve cord is marked with a thick arrow (b), scale bar 10µm;
(c) ***Bilharziella polonica.*** 5-HT-IR fibres extending towards the ventral sucker (thin arrows), scale bar 10µm. Note the nerve cells near the ventral sucker (thick arrows). Inset: the pattern of TRITC-phalloidin labeled F-actin in the ventral sucker;
(d) ***Sphaerostomum globiporum.*** 5-HT-IR cells near the ventral sucker (arrow), scale bar 10µm;
(e, f) ***Moliniella anceps.*** FMRFamide-IR (e) and 5-HT-IR (f) fibres in the oral sucker (thin arrows), scale bar 20µm. The nerve cells are marked by thick arrows (f);
(g, h) ***Himasthla elongata.*** (g) 5-HT-IR fibres extended to the ventral sucker (arrows). Inset: the pattern of TRITC phalloidin labeled F-actin in the ventral sucker. (h) FMRFamide-immunoreactivity in the oral sucker (arrow), scale bar 20µm.

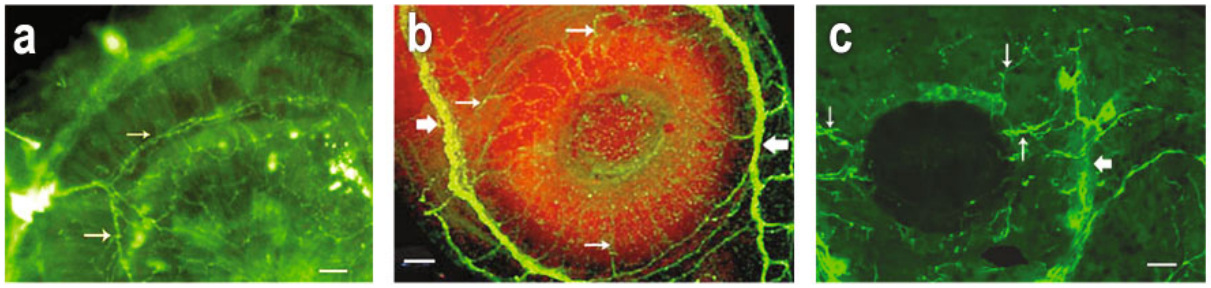

Fig. 5(a – c). The serotoninergic and FMRFamidergic components of the nervous system in the attachment organs of metacercariae.
(a, b) ***Leucochloridiomorpha lutea***. 5-HT-IR (a) and FMRFamide-IR (b) nerve fibres in the ventral sucker (thin arrows).
The main nerve cords are marked with thick arrows. Scale bar 50 μm;
(c) ***Cotylurus sp.*** 5-HT-IR nerve fibres extending towards the ventral sucker (thin arrows) from the main nerve cord (thick arrow), scale bar 10μm.

1991; Mair *et al*., 2000; Fairweather *et al*., 1987; Gustafsson *et al*., 1987; Magee *et al*., 1989; Tolstenkov *et al*., 2010). There are also data indicating the existence of nerve fibres containing these neurotransmitters in the attachment organs of the larvae (cercariae and metacercariae) of *Echinostoma caproni*, *Cercaria emasculans*, *Neoastiotrema trituri*, *Echinostoma revolutum*, *Cathaemasia hians*, *Paramphistomum cervi*, *Psilohasmus oxyurus*, *Cercaria parvicaudata*, *Diplostomum sp.*, *Cotylurus erraticus*, *Apatemon cobitidis proterorhin*, *B. gracilescens*, *O. viverini*, *M. anceps* and *O. felineus* (Šebelova *et al*., 2004; Pan *et al*., 1994; Barton *et al*., 1993; Lecsanboon *et al*., 2012; Stewart *et al*., 2003a, b; Tolstenkov *et al*., 2010, 2012a, b).

Our study was performed on adults and larval stages of different trematode species and confirmed the presence of 5-HT- and FMRFamid-ergic nerve structures in the oral and ventral suckers of adults (eight species) and larvae (ten species) from various taxonomic groups, including the most ancient subclass, Aspidogastrea. The results not only revealed for the first time the innervations of the attachment organs in trematode species not studied before, but also confirmed and expanded the data already available on this issue for several species (*M. anceps*, *B. polonica*, *T. szidati*, *C. parvicaudata*, *O. felineus*). It can be concluded that the presence of studied neuromediators in the innervations of the attachment organs is characteristic for phylum Trematoda.

We found that FMRF-immunoreactivity in the fixation organs of some trematodes studied herein was more intensive than 5-HT-immunoreactivity. This is true for *Opisthorchis felineus* (adults), metacercariae of *Leucochloridiomorpha lutea* and cercariae of *Cryptocotyle lingua*. A question on possible differences in localization pattern of each neurotransmitter between the adult trematodes and their larvae is an interesting one. Based on own data and existing literature it is difficult to perform such comparative analysis as it requires simultaneous staining and investigation of the different life stages in one trematode species, which were not available at the same time. In some cases the immunoreactivity to serotonin or FMRFamide was not enough pronounced (or even absent) in attachment organs, what could be due to a limited quality of samples. However, the presence of nerve structures could not be ruled out.

In general, in flatworms serotonin acts as an excitatory neurotransmitter. The exogenous 5-HT can induce or enhance the motility of muscle strips or contractions of individual musle fibres prepared from monogeneans, trematodes, and cestodes (reviewed by Halton & Maule 2004). FaRPs have also been shown to be myoexcitatory in a concentration-dependent manner when were applied exogenously to isolated muscle fibers or muscle-strips from free-living *Bdelloura candida* and *Procerodes littoralis* (Johnston *et al*., 1996; Moneypenny *et al*., 2001) and parasitic flatworms *Schistosoma mansomi*, *Fasciola hepatica*, *Mesocestoides corti* (Day *et al*., 1994; Marks *et al*., 1997; Hrčkova *et al*., 2002). It was showed that FaRPs are acting through different types of receptors involving different second messenger pathways than was shown for serotonin (Zamanian *et al*. 2011; Patocka *et al*. 2014). The innervations of the copulatory organ and genital tracts by FMRF-IP nerve fibres have also suggested a role of FaRPs in the reproductive system of platyhelminths (Gustafsson *et al*. 2002).

In summary, present study revealed the pattern of innervations of the attachment organs with serotoninergic and peptidergic nerve structures in adults and larvae of trematodes from various taxonomic groups, which have different life cycles, hosts and localization within them. The innervations of the oral and ventral suckers in trematodes imply an important role of the nervous system, namely its serotoninergic and peptidergic components in the regulation of the function of trematodes' adhesive organs. Based on our observations and similar studies on various trematode species, we can conclude that the innervations of trematode fixative organs with serotoninergic and peptidergic (FaRPs) neurotransmitters are widely represented (if not universal) characteristic of this class of parasitic flatworms.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Modulation of TLR2 and TLR4 in macrophages following *Trichinella spiralis* infection

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Summary

Parasitic helminthes can suppress and/or regulate the host immune response to allow long-term survival and chronic infection where toll-like receptors (TLRs) expressed on macrophages play essential roles in response to parasitic infection. Semi-quantitative PCR and flow cytometry studies about the modulation of TLRs and cytokine profiles in macrophages following *T. spiralis* infection were performed. TLRs, MyD88 and NF-κB were up-regulated by *T. spiralis* infection and essential to the parasite life cycles. Cytokines profiles (IL-6, IL-10, IL-12, TNF-α) were modulated during *T. spiralis* infection. Results suggest that *T. spiralis* infection may regulate the expression of TLR4 on macrophages and TLR4/MyD88/NF-κB signaling pathways. This study provides further insights into the mechanisms of TLR-mediated post-inflammatory response during *T. spiralis* infection.
Keywords: *Trichinella spiralis*; Toll-like receptor; Helminth; Immune modulation

Introduction

Trichinellosis is a wide-spread foodborne zoonosis initiated by the ingestion of raw or poorly-cooked meats containing infectious *Trichinella spiralis (T. spiralis)* larvae. Trichinellosis is considered as a re-emerging disease and it has been reported worldwide. Trichinellosis is not only an important source of public health hazard but also an economic problem in animal production and food safety (Yang *et al*., 2010; Chen *et al*., 2012; Li *et al*., 2012; Yadav *et al*., 2012; Cui *et al*., 2013). Infection with *T. spiralis* represents a great challenge for the host immune response. It is due to parasite life cycle of the parasite which is accomplished in one host. The larvae are released from infective capsule-like cysts and undergo maturation process to reach the adult reproductive stage within the intestine. The newborn larvae released from the adult females disseminate through the host by the circulation. Larvae finally enter the skeletal muscles and develop into infective encysted muscle larvae that are essential for transmission (Kang *et al*., 2012; Riva *et al*., 2012). In order to complete the life cycle and survive in the host, *T. spiralis* influences the development of immune-regulatory mechanisms essential for immune tolerance (Radovic *et al*., 2015). However, the nature of the relationship between *T. spiralis* and the host organism, as well as mechanisms and signals that control the immune response, have been understood or investigated not sufficiently.

Helminthes have developed different evasion and suppression mechanisms what enable the establishment of infection with lowest possible damage to the host (Motran *et al*., 2017). The immune response caused by *T. spiralis* is characterized as mixed Th1/ Th2 type of immune response where during the intestinal phase, the Th1 response predominate and subsequently a Th2 response will follow (Ilic *et al*., 2012). Expulsion of the gastrointestinal adult is associated with a prominent mucosal mastocytosis mediated by a Th1 response involving the production of cytokines such as IL-4, IL-5 and IFN-γ (Blum *et al*., 2013; Liu *et al*., 2013; Thrasher *et al*., 2013). The immunological response to *T. spiralis* muscle invasion

* – corresponding author

is primarily characterized by a Th2 phenotype, in which cells collected from cervical lymph nodes produce IL-5, IL-10, IL-13 and IFN-γ after the stimulation with somatic larval antigens (Bruschi *et al*., 2011). IL-10 controls the level of inflammation induced by *T. spiralis* especially during the chronic phase of infection (Aranzamendi *et al*., 2013). Excretory-Secretory (ES) products from different stages of *T. spiralis* infection can modulate macrophages function *in vitro* by inhibiting pro-inflammatory cytokine production (Yu *et al*., 2013) and the muscle larvae ES products have the properties that regulate the immune response by its suppressive effect on dendritic cells (DCs) maturation (Aranzamendi *et al*., 2013; Gruden-Movsesijan *et al*., 2011). *T. spiralis* infection alters the immune response via increased production of both IL-4 and IL-10 and decreased production of IFN-γ and IL-17 (Gruden-Movsesijan *et al*., 2010).

Pattern recognition receptors such as Toll-like receptors (TLRs) may regulate the response of DCs and macrophages, as well as the other immune cells of the innate immune system such as mast cells (Yu *et al*., 2013; Langelaar *et al*., 2009). *In vitro, T. spiralis* ES antigens can suppress DCs maturation but this effect depends on the type of lipopolysaccharide (LPS) used to activate these cells (Langelaar *et al*., 2009). Studies showed that the suppressive effect of ES on DCs maturation is restricted to TLR4 using different TLR agonists and the ES products can also interfere with the expression of several genes related to the TLR-mediated signal transduction pathways (Aranzamendi *et al*., 2012). Induction of inflammatory cytokines by LPS is mediated by nuclear factor-κB (NF-κB). The ES products may modulate the macrophages activities *in vitro* and the roles of ES products in the LPS-induced nuclear translocation of NF-κB are being investigated. Regulation of inflammatory cytokine induction via the NF-κB pathways is an important mechanism in parasite infection (Bai *et al*., 2012). Despite many immunomodulatory investigations concerning helminthes or their products, very little is known about the TLRs mechanisms and signals that control the immune response and systemic cytokine response *in vivo*. In this study, cytokine profiles in serum and macrophages were examined from mice infected with *T. spiralis*. The expression of TLR4 was determined by flow cytometry analysis and macrophages were analysed with semi-quantitative PCR during *T. spiralis* infection. In addition, the expression of MyD88 (myeloid differentiation primary-response gene 88) and NF-κB was measured by Western blot analysis. The different stages of *T. spiralis* had different impacts on the expression of TLRs and related signaling molecules.

Materials and Methods

Animal and parasite infection

Male BALB/C mice, 6 – 8 weeks old, purchased from the Experimental Animal Center of Harbin Veterinary Research Institute of Chinese, Academy of Agricultural Sciences (CAAS) were infected orally with 500 *T. spiralis* larvae (isolate code: ISS3; original host: domestic pig of Poland). The muscle larvae were isolated as described previously (Mido *et al*., 2012). The blood serum was collected from the mouse eye socket at 0, 4, 7, 14, 21 and 28 days post infection (p.i.). The group sizes consisted of three mice per group per each time points.

Abdominal macrophages isolation

Infected mice were sacrificed at 4, 7, 14, 21 and 28 days p. i. and the uninfected mice were used as control. The RPMI 1640 medium (Gibco, Grand Island, USA) was instilled into the mouse abdomen and cells were concentrated by spinning (3000 g, 10 min) and by subsequent supernatant removal. Tris-NH_4CL was added to lyze the red cells and macrophages were cultured in RPMI 1640 supplemented with 10 % fetal calf serum (Sigma, Saint Louis, USA) on 24-well plates and maintained at 37 °C in 5 % CO_2 for 2 hr. After the incubation, the non-adherent cells were removed by washing and adherent cells were re-supplemented again in RPMI 1640 with 5 % FCS. Afterwards counted and checked for viability. Macrophages were then seeded into 24-well plates and allowed to adhere for another 24 hr. Then the cells were collected and flow cytometry analysis was performed. Two treatments were carried out on macrophages. Cells were either LPS untreated or treated with LPS for the first 12 h (final conc., 100 ng/ml). The cells and cultured and supernatants were collected and stored at -80 °C until the ELISA was performed.

Cytokine and NO level analyses

The levels of IL-6, IL-10, IL-12, TNF-α and NO in serum and cultured supernatants were determined by cytokine ELISA kit (RD Systems, Minneapolis, USA) according to the manufacturer's instructions. The absorbance of the wells was read at 450nm on microplate reader (BIO-RAD 680, Hercules, USA). Cytokine and NO concentrations in examined samples were calculated according standard curves generated with known concentrations of cytokines (BD Pharmingen, San Diego, USA). Results are expressed in picograms per mililiter ($pg.ml^{-1}$).

Semi-quantitative RT-PCR

Total RNA was extracted, from the samples of macrophages at different day p. i., by TRIZOL RNA extraction kit (Invitrogen, Vienna, Austria) and according to the manufacturer's instructions. The RNA from each sample was reverse-transcribed to cDNA with Reverse Transcription System (Promega, Madison, USA). The PCR primers were designed by use of the Primer-BLAST tool at the NCBI website. The forward primer of the TLR4 was 5'-tcacctgatacttattgctgg-3' and the reverse primer was 5'-agttgccgtttcttgttct-3' (GenBank accession number NM_021297.2). A pair of primer for TLR2 (5'-ctgagaatgatgtgggcgt-3' and 5'-cttggtgttcattatcttgcg-3'; GenBank accession number AF165189) was developed. Levels of target genes were normalized to that of β-actin as a housekeeping gene (5'-ctgtccctgtatgcctctg-3' and 5'-atgtcacgcacgatttcc-3'; GenBank accession number NM_007393). Amplification of the

fragments at 538 bp TLR4 and 426 bp TLR2 and 218 bp β-actin was carried out by PCR. The PCR reaction was conducted under the following conditions: an initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 53.5 °C, 55 °C and 55 °C for 30 s and elongation at 72 °C for 30 s, and a final extension at 72 °C for 10 min (Mini Cycler® Peltier Thermal cycler, MJ Research, Waltham, USA). The PCR products were analyzed by electrophoresis on 1 % agarose gel and the density of the PCR gene mRNA was divided by that of the density β-actin to obtain a normalized value for target gene expression.

Flow cytometry

Abdominal macrophages were washed and re-suspended in PBS and cells were phenotyped for the expression of the CD282/TLR2 and CD284/TLR4 cell surface markers. 1×10^6 cells were incubated in the dark at room temperature for 1 hr with PE-conjugated anti CD284 antibody (eBioscience, San Diego USA). Then the cells were washed twice with PBS and flow cytometry analysis was carried out on a FACScalibur (BD, San Jose, USA) equipped with FACS Diva 6.0 software.

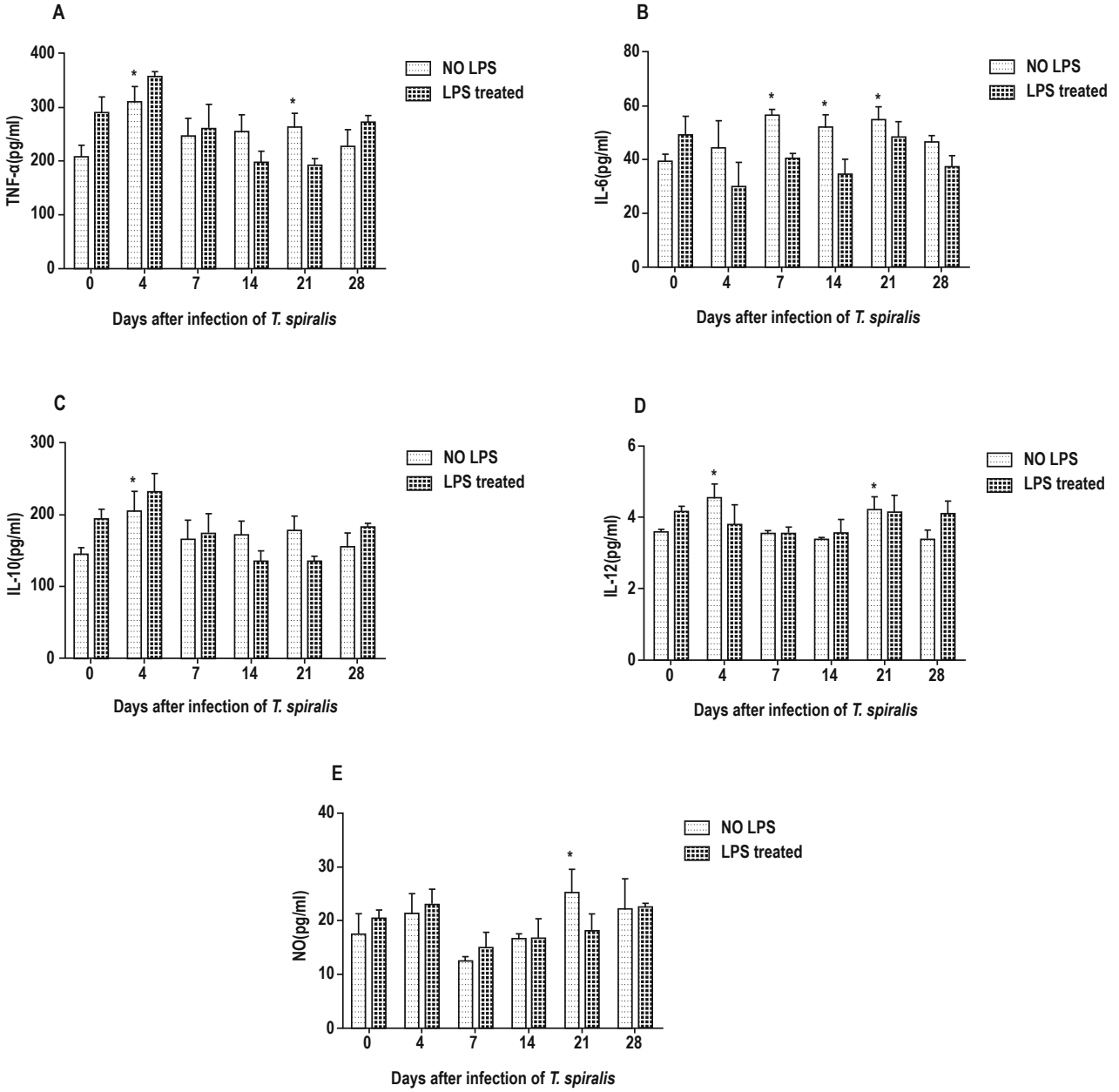

Fig. 1. Cytokine profile in the macrophages supernatants after infection with *T. spiralis* at the indicated time points. Cells were treated with or without LPS (final conc., 100 ng/ml) for 12 h. No cytokines production was detected in control group without *T. spiralis* infection (data not shown). A – E: TNF-α, IL-6, IL-10, IL-12 and NO levels in the cell supernatant were measured by ELISA.

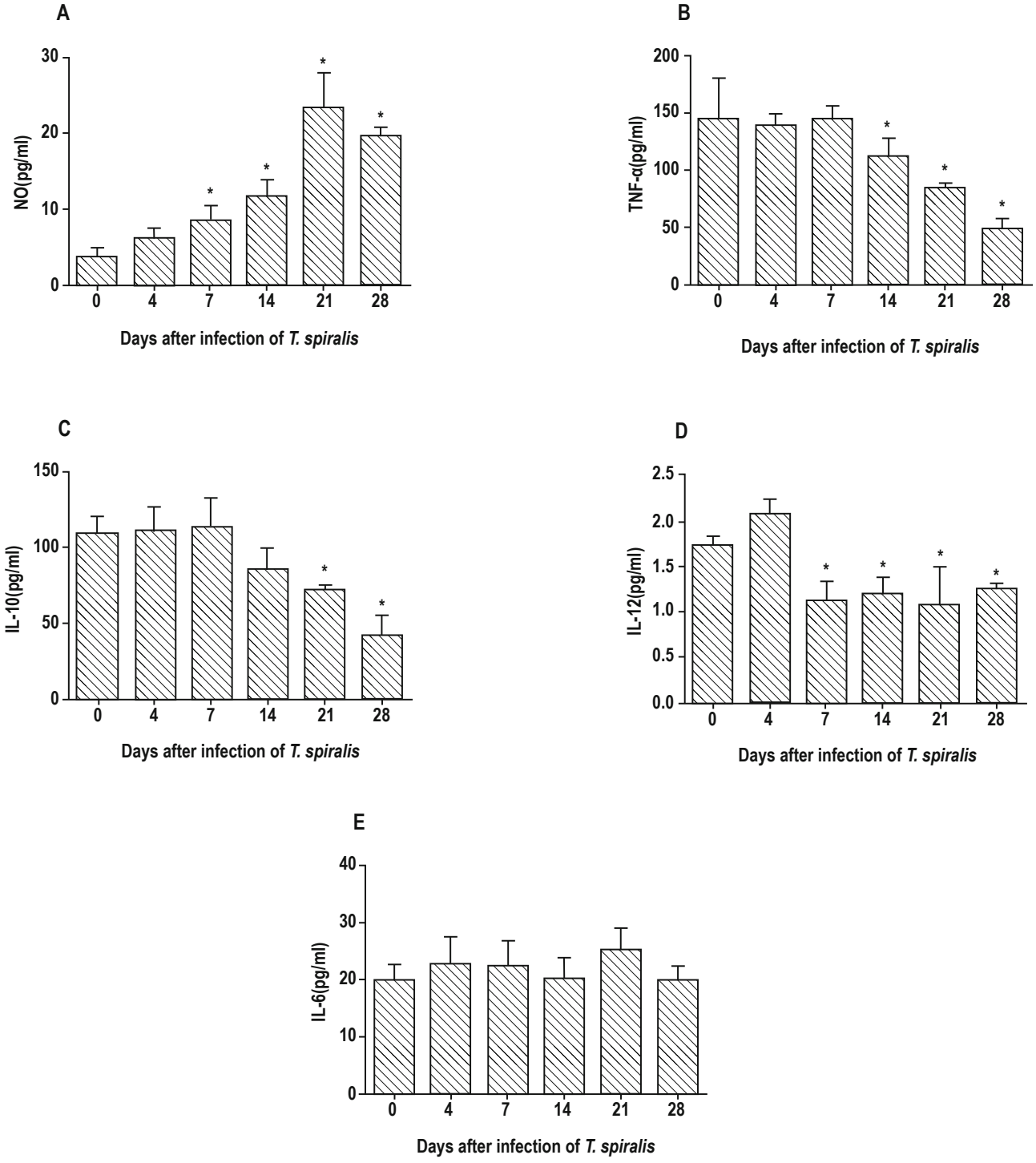

Fig. 2. Cytokine profile in the serum after infection with *T. spiralis* at the indicated time points. The serum was separated from blood taken from the mouse eye socket. No cytokines production was detected in control group without *T. spiralis* infection (data not shown). A-E: NO, TNF-α, IL-10, IL-12 and IL-6 levels in the serum were measured by ELISA.

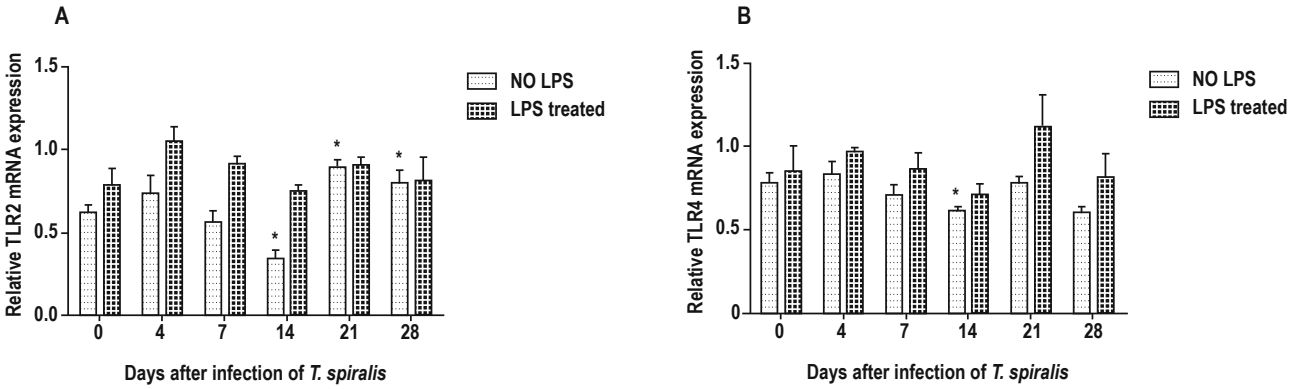

Fig. 3. The TLR2 (A) and TLR4 (B) mRNA expression in macrophages in mice after infection with *T. spiralis* at the indicated time points by RT-PCR. Cells were either left untreated with LPS for 12 h or treated for the same time with LPS (final conc., 100 ng/ml) for 12 h. A total RNA was purified from cells and TLR mRNA were assayed by semi-quantitative RT-PCR.

Western blot analysis

Macrophages were collected and proteins were extracted according to the manufacturer's handbook (KeyGEN BioTECH, Nanjing, China). Protein concentrations in each sample were determined by Bradford Protein Assay Kit (KeyGEN BioTECH, Nanjing, China). The proteins were then separated with 10 % SDS-PAGE and transferred on nitrocellulose membrane, which was blocked overnight with 5 % (w/v) non-fat dry milk in TBST (20 mmol/l Tris-HCl (pH 7.6), 150 mmol/l NaCl and 0.02 % Tween 20). Membranes were then washed three times for 10 min in TBST and incubated for 1 hr at 37 °C with rabbit polyclonal anti-MyD88, anti-NF-κB and anti-β-actin primary antibodies (Bioss, Beijing, China). In addition, anti-β-actin was used to detect β-actin expression as a quantitative control. After being washed three times for 10 min in TBST the membranes were incubated for 1 hr at RT with horseradish peroxidase (HRP)-conjugated secondary antibody. The membranes were then washed again three times in TBST and protein bands were visualized with the ECL enhanced chemiluminescent (HaiGene, Harbin).

Statistical analysis

Results are presented as mean ± SD. Significance of the differences between experimental and control groups were calculated using Student's *t* test. In all cases, a *p* value less than 0.05 was considered to be statistically significant.

Ethical Approval

All animal husbandry and experimental procedures were performed in accordance with the Chinese Animal Management Ordinance (People's Republic of China Ministry of Health document No.55 in 2001). The research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

Results

Cytokine measurement

The expression of TNF-α, IL-6, IL-10, IL-12 and NO in the LPS-stimulated macrophages from the infected mice was detected

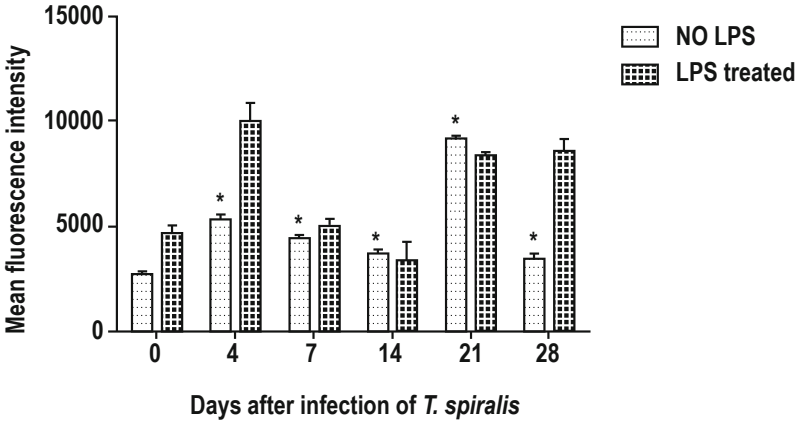

Fig. 4. The MFI of TLR4 in macrophages in mice after infection with *T. spiralis* at indicated time points by flow cytometry. Flow cytometry was carried out and analyzed on a FACScalibur (BD, ADD CITY, USA) with FACS Diva 6.0 software.

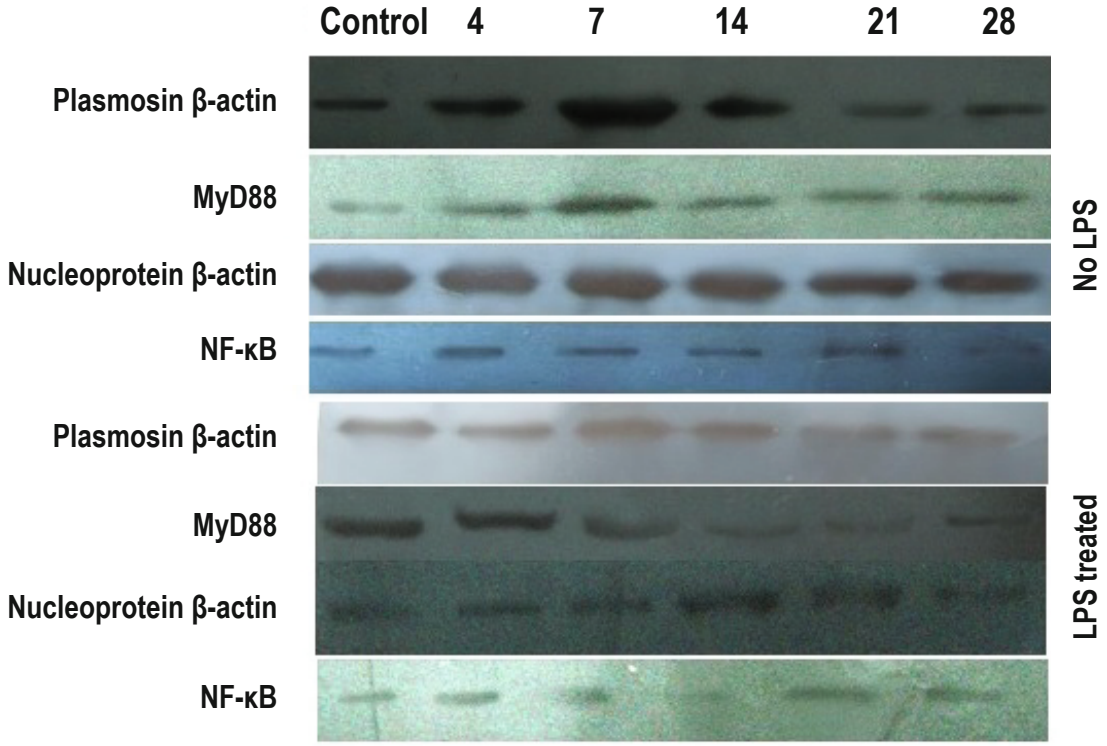

Fig. 5. MyD88 and NF-κB expression in macrophages after infection with *T. spiralis* at indicated time points by western blot. Cells were treated with LPS (final conc., 100 ng/ml) for 12 h. The protein was detected in control group without *T. spiralis* infection. Protein samples were analyzed by western blot with phospho-specific antibodies. 4, 7, 14, 21 and 28: The time points (days) after the infection of *T. spiralis*.

by ELISA. TNF-α (Fig. 1 A) levels were up-regulated in non-LPS treated macrophages at days 14 and 21 p. i.. Higher IL-6 (Fig. 1 B) expression readings in cell culture supernatants that were detected at days 7 – 21 p. i., while the IL-10 (Fig. 1 C) and IL-12 (Fig. 1 D) levels were up-regulated only at day 4 p. i.. There were no differences in the cytokine production between LPS-treated macrophages and control. Upon the LPS stimulation, there were differences in TNF-α, IL-6 and IL-10 but not in IL-12 and NO (Fig. 1 E) between the groups at days 14 – 21 p. i.. The amount of NO (Fig. 2 A) significantly increased in the serum after *T. spiralis* infection, meanwhile the levels of TNF-α (Fig. 2 B), IL-10 (Fig. 2 C) and IL-12 (Fig. 2 D) decreased significantly. However, no changes were detected in IL-6 expression (Fig. 2 E).

Quantification of TLRs expression

To determine whether the TLRs expression was differently regulated, the mRNA of TLRs was determined in macrophages of *T. spiralis*-infected mouse *in vitro*. The quantification of TLRs mRNA was measured by normalization to β-actin. The TLR2 expression in LPS-stimulated macrophages was up-regulated when compared with naive cells (Fig. 3 A). There was no difference in the TLR2 expression between macrophages with or without LPS stimulation, except day 21 p. i. (Fig. 3 B). There was no difference in the TLR4 expression between the LPS-stimulated and non-LPS stimulated cells on day 14 p. i Flow cytometry showed that the TLR4 expression decreased only at day 21 p. i. in the LPS-stimulated macrophages when compared with naive cells (Fig. 4).

Expression of MyD88 and NF-κB by Western blot analysis

It is known that MyD88-dependent pathway is one of the TLRs signaling pathways mediated universally by all TLRs. The expression of MyD88 and NF-κB involved in activating or regulating TLRs signaling was examined on macrophages at indicated time points after *T. spiralis* infection. The MyD88 and NF-κB expressed on macrophages were similar to the TLR4 mRNA expression pattern (Fig. 5). The expression of MyD88 was up-regulated on day 4 and 7 p.i. at intestinal phase. So we inferred that the intestinal adult may activate the MyD88. In addition, the expression of MyD88 was up-regulated by the muscle larvae. Relative MyD88 and NF-κB expression in no LPS show statistical significance at day 21 and 28 p.i. (Fig. 6 A, B). Those days represent chronic infection. The exact mechanism needs further investigation.

Discussion

Helminthes parasites are of considerable medical and economic importance. Studies on the immune response against helminthes are of great interest by reason of understanding the interactions

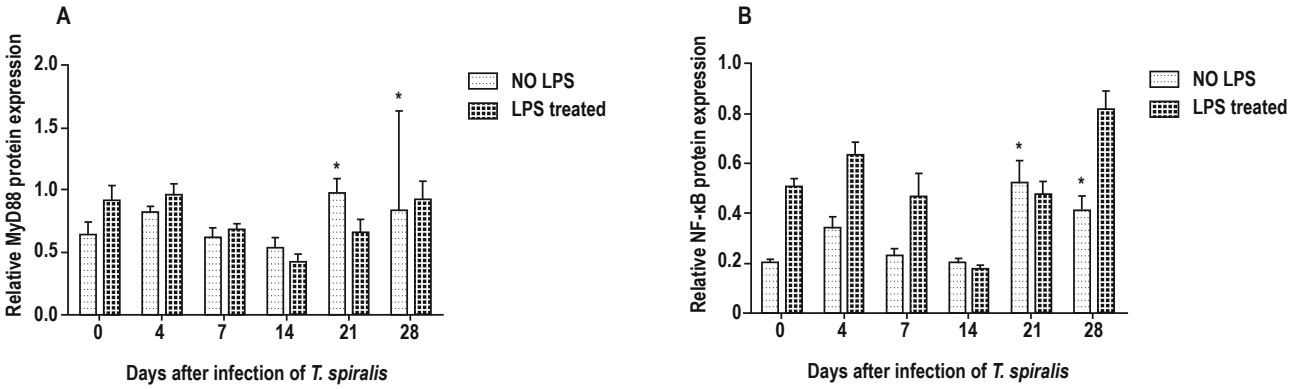

Fig. 6. The expression of MyD88 (A) and NF-κB (B) in macrophages after infection with *T. spiralis* at the indicated time points. Cells treated with or without LPS (final conc., 100 ng/ml) for 12 h. The protein concentrations were determined by using the Bradford Protein Assay Kit. Expression is normalized to β-actin.

between host immune system and parasites (Moreau *et al*., 2010). In order to maintain their life cycle, helminthes parasites can modulate the host's immune response that would enable their long-term survival within the host. Parasites are able to persist in the host and are mainly responsible for chronic infection despite a strong immune response developed by the parasitized host (Ilic *et al*., 2011). The pathogenic organisms (virus, bacteria and parasite) can activate antigen presentation cells (APCs) primarily through pattern recognition receptors – TLR recognition and signaling (Yu *et al*., 2013; Kim *et al*., 2015). The antigen component of *T. spiralis* may possibly serve as a ligand of TLR4 and ES antigen suppressed cytokine production and expression of co-stimulatory markers on DCs induced by TLR4. It was found that the expression of CD40, CD80 and CD86 of the DCs surface and cell factors (IL-6, IL-10 and TNF-α) was decreased (Langelaar *et al*., 2009).

Previous study has demonstrated that macrophages play crucial roles in host immune responses against various pathogens (Bai *et al*., 2012). Given the recent advances in activated macrophages under the control of Th2-dependent cytokines expression in helminthes infection (Bruschi *et al*., 2008), the expression levels of TLR2 and TLR4 on macrophages in *T. spiralis* infection was investigated. Our results showed that the mRNA expression of macrophages TLRs *in vivo* was differently regulated throughout *T. spiralis* infection. The TLR2 expression was increased only in the LPS-stimulated macrophages but not in non- stimulated macrophages at day 4 p. i.. The expression levels of TLR2 and TLR4 decreased gradually from day 7 p. i. Probably due to increased antigens production induced by newborn *Trichinella* larvae what suggest that newborn larvae have extensive inhibitory effects on host immune response. The expression was lowest at day 14 p. i. and increased gradually up to day 21 thus contributing to the tolerance elimination and formation of the cysts in muscles. These results suggest that *T. spiralis* ES products at each stage of infection are potential factors for regulation of host immune response by modulation of TLRs expression and signal pathways in macrophages.

Intestinal DCs and macrophages retain regulatory mechanisms what prevent excessive inflammation, and there is an active suppression of pro-inflammatory cytokine production by regulatory cytokine such as IL-10 (Pulendran *et al*., 2010; Beiting *et al*., 2007). Macrophages stimulated by LPS differentiate into classically activated macrophages and release a number of immune mediator molecules (IL-12, IL-6, TNF-α, nitric oxide – NO), which play critical roles in initiation and modulation of the host immune responses to parasite infections (Bai *et al*., 2012; Noel *et al*., 2004). Further we investigated the dynamic changes of cell factors from different stages of *T. spiralis* in serum and macrophages. TNF-α, IL-6, IL-10 and IL-12 were decreased in LPS-stimulated macrophages, as well as TNF-α, IL-6, IL-10 and IL-12 in serum. Results are comparable with those of Bai and colleges who found that the ES products from different stages of *T. spiralis* infection significantly suppressed the production of pro-inflammatory TNF-α, IL-Iβ, IL-6 and IL-12 in LPS-stimulated macrophages *in vitro* (Bai *et al*., 2012). NO was reported to play an important role in induction of intestinal physiology and inflammation during *T. spiralis* infection (Lawrence *et al*., 2000). The regulation of NO release is associated with iNOS release, and induced macrophages can produce the enzyme NOS2 (an enzyme responsible for the production of nitric oxide) which transforms L-arginine to nitric oxide, responsible for parasite damage (Bruschi *et al*., 2011). Our results showed that the NO expression was increased in serum and decreased in LPS-stimulated macrophages at day 21 p. i.. Our results are consistent with the findings that ES products had a suppressive effect on LPS-stimulated iNOS expression in macrophages (Bai *et al*., 2012).

The NF-κB signaling pathway regulates LPS-stimulated pro-inflammatory response in macrophages. The ES products of helminthes have been found to inhibit NF-κB activation (Puneet *et al*., 2011). *Trichinella* ES products are able to inhibit the LPS-stimulated nuclear translocation of p65 and also reduce ERK1/2 and p38 MAPK phosphorylation (Bai *et al*., 2012). TLR4 signaling involves two main intracellular pathways. One is MyD88-dependent pathway which mediates the production of pro-inflammatory cytokines and second is MyD88-independent or TRIF pathway which medi-

ates the up-regulation of co-stimulatory and MHC II molecules on DCs (Du *et al*., 2014; Scalfone *et al*., 2013). Here we found that the expression levels of NF-κB and MyD88 was similar to the TLR4 expression in LPS-stimulated macrophages *in vivo*. The TLR4 expression changes induced by *T. spiralis* infection could activate the TLR4/MyD88/NF-κB signaling pathway and also effect the secretion of inflammatory cytokines in macrophages, what could participate in immune suppression.

In summary, we confirmed that experimental *T. spiralis* infection in mice could modulate the host immune response. Different stages of infection had different effects on cytokine profiles, as well as the TLRs and related signaling molecules in macrophages. The expression of TLR2 and TLR4 were modulated at different stages of *T. spiralis* infection. The cytokine levels were regulated through TLR4-mediated signaling pathway, suggesting that TLR4 modulated the immunosuppression of the host. This study provides fresh insights into the mechanisms of TLR-mediated post-inflammatory response during *T. spiralis* infection.

Conflict of Interest

Authors state no conflict of interest. Authors have no potential conflict of interest pertaining to this submission to Helminthologia.

Acknowledgments

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Digital image analysis to estimate the minimum number of *Eurytrema coelomaticum* eggs in the uterus of adult specimens

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Article info

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Summary

This study was carried out to estimate the minimal number of eggs present in adult *E. coelomaticum* uterus. Samples were collected during post-mortem inspection and were submitted to light microscopy (bright field). The length, width, the total area of the parasite, uterus, and eggs were measured. The ImageJ software was used to calculate the area of the different parameters analyzed in this study. It was possible to observe that the uterus corresponds on average to 51.9 % of the total area of the parasite (ranging from 45 to 64 %). The number of eggs present in the uterus of parasites ranged from 5,946 to 15,813. To estimate the number of eggs three scenarios were considered, where the first taken into account the number of whole eggs observed in the image. In the second way to estimate the number of eggs, all the structures were considered (whole eggs and fractions that could be delimited) and compared with manual counting. Finally, in the last scenario, was considered an occupancy rate of 100 % of the uterine area per eggs, since there are overlapping eggs and these cannot be correctly delimited and accounted for. This study describes an important tool for quantifying eggs in a nondestructive manner and aggregate information until then is not explained by other works.

Keywords: Trematoda; Dicrocoeliidae; ImageJ

Introduction

The digenetic trematode *Eurytrema coelomaticum* (Giard & Billet, 1892) Looss, 1907 is parasite of pancreatic ducts of ruminants and are endemic in several regions of the world, including countries of South America, Europe and Asia (Sakamoto *et al*., 1981; Ilha *et al*., 2005; Sakamoto & Oikawa, 2007; Quevedo *et al*., 2013). In Brazil, this helminth is widely distributed and commonly found in slaughterhouses in several states (Azevedo *et al*., 2004; Bassani *et al*., 2006) The life cycle is heteroxenic, requiring two intermediate hosts and another definitive. The primary intermediate host is the mollusk *Bradybaena similaris* (Férussac, 1821) (Gastropoda, Xanthonychidae) (Ilha *et al*., 2005). In Brazil, the second intermediate host is a grasshopper of the genus *Conocephalus* (Thunberg, 1815) (Orthoptera, Tettigoniidae) and in some regions of Asia the cricket belongs to the genus *Oecanthus* (Serville, 1831) (Orthoptera, Gryllidae) (Ilha *et al*., 2005; Pinto & De Melo, 2013, 2016). Finally, the definitive hosts become infected upon ingestion of infected arthropods with metacercariae when feeding on grazing, thus completing the cycle (Bassani *et al*., 2007).

The eurytrematosis is clinically asymptomatic, but is a silent disease which causes losses in milk and meat production. When it considered in a little number of animals these losses are diluted and not clearly visible. But, when you consider a loss of 1 l of milk or 1 kg of meat production per animal in a cattle with 10,000 animals this is very significant in an economic point of view. Although

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it is commonly found infecting herds in Brazil, there are no calculations about the impact of these organisms on national livestock production, with estimated costs varying from 1 to 3 % of the total amount (Ilha *et al*., 2005). Is associated with hyperplasia and hypertrophy of the pancreas and bile ducts, as well as fibrosis and partial or total obliteration of the pancreatic ducts when in massive infections (Rachid *et al*., 2011; Figueira *et al*., 2014; Schwertz *et al*., 2016).

The World Association for the Advancement of Veterinary Parasitology (WAAVP) recommends performing count of eggs per gram of faeces (EPG) to assess the intensity of parasitic infection of various trematodes species (Wood *et al*., 1995). Observation of the EPG levels is largely recommended and is historically used as a diagnostic and monitoring tools in the control of parasites of veterinary importance, supporting in the treatment and mainly in prophylaxis (Bassani *et al*., 2007; Wood *et al*., 1995). Although it is an important tool for the evaluation of worm eggs, in general, the technique presents values underestimated and can in some cases compromise the treatment and the prophylaxis, fact that reinforces the importance of the present study. Gassó *et al*. (2015) have shown that depending on the location where the parasite is situated the eggs prevalence has decreased due to the routes through which the eggs must pass until reaching the intestine. They further cite that accuracy has declined over time and therefore it is recommended that samples be processed within 5 days after collection.

The *E. coelomaticum* eggs are oval with a coloration ranging from yellowish to brownish when immature and brown when fertile (Rachid *et al*., 2011; Pinheiro *et al*., 2015). Egg size ranges from 39 to 50 µm in length by 20 to 32 µm in width (Yamamura, 1989; Mohanta *et al*., 2015; Pinheiro *et al*., 2015).

Yamamura (1989) analyzing the oviposture of *E. coelomaticum* (*in vitro*) observed an average daily of 641 eggs. Brandolini and Amato (2001) testing different physiological media observed that Locke solution presented the best oviposture rate with an average of 746 ± 139 eggs, followed by Earle solution with 304.6 ± 154.7 and finally the saline solution to 0.85 % with 194.5 ± 132 eggs. About this information, there are no records in the literature of estimate number of eggs present in the uterus of *E. coelomaticum*.

The use of techniques such as the analysis of digital images makes possible the estimation of eggs present in an adult animal and thus aggregates information about its fecundity and epidemiology (Rosati *et al*., 2015; Mains *et al*., 2008). In this work, we describe methodologies that were used to calculate the mean uterine size, the proportion of the organ in relation to the total size of the animal, as well as to estimate the number of eggs of *E. coelomaticum* according to the occupancy rate of the parasite uterus.

Materials and Methods

Samples of *E. coelomaticum* were obtained during the post-mortem inspection at the slaughterhouse in the city of Campo Belo, Minas Gerais, Brazil. The samples were processed at the Labo-

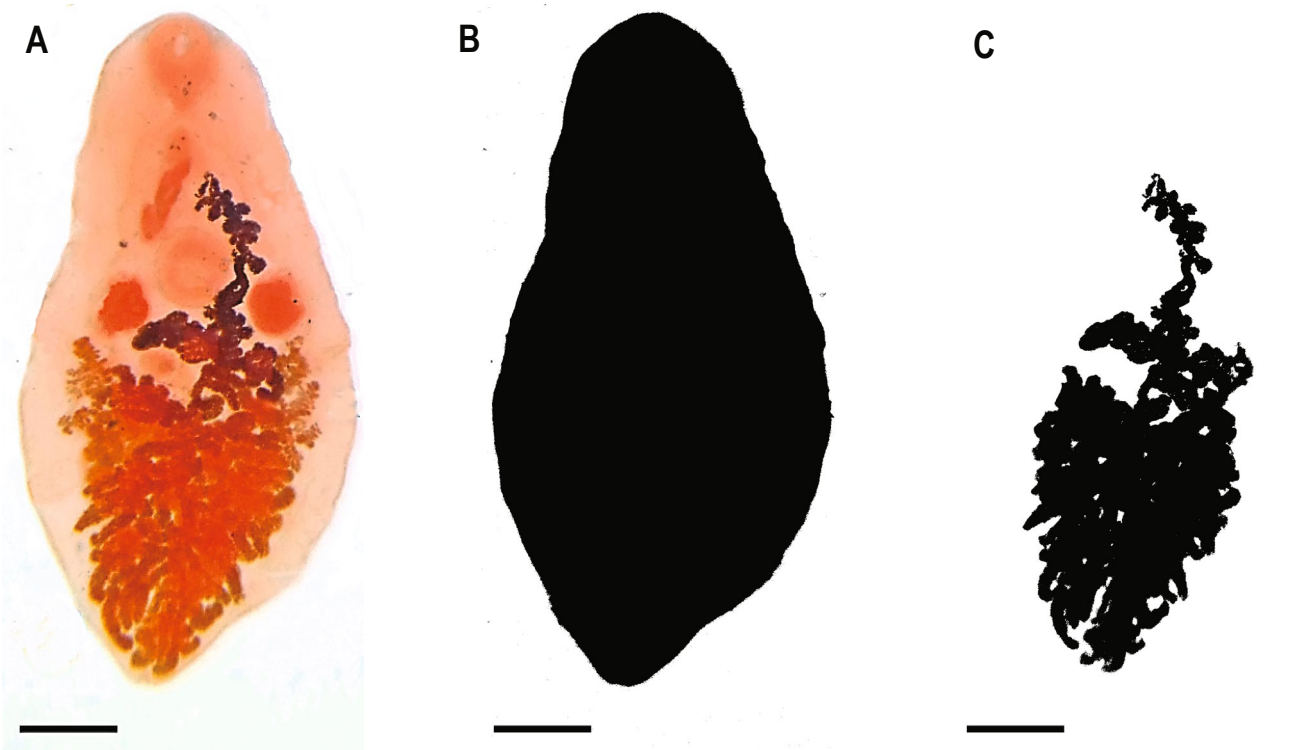

Fig. 1. The binarization process to measure the surface area. (A) general view from *Eurytrema coelomaticum.* (B) binary image after transformation in ImageJ software showing the total area of the parasite. (C) uterus area after the binarization process. Scale bar 1 mm

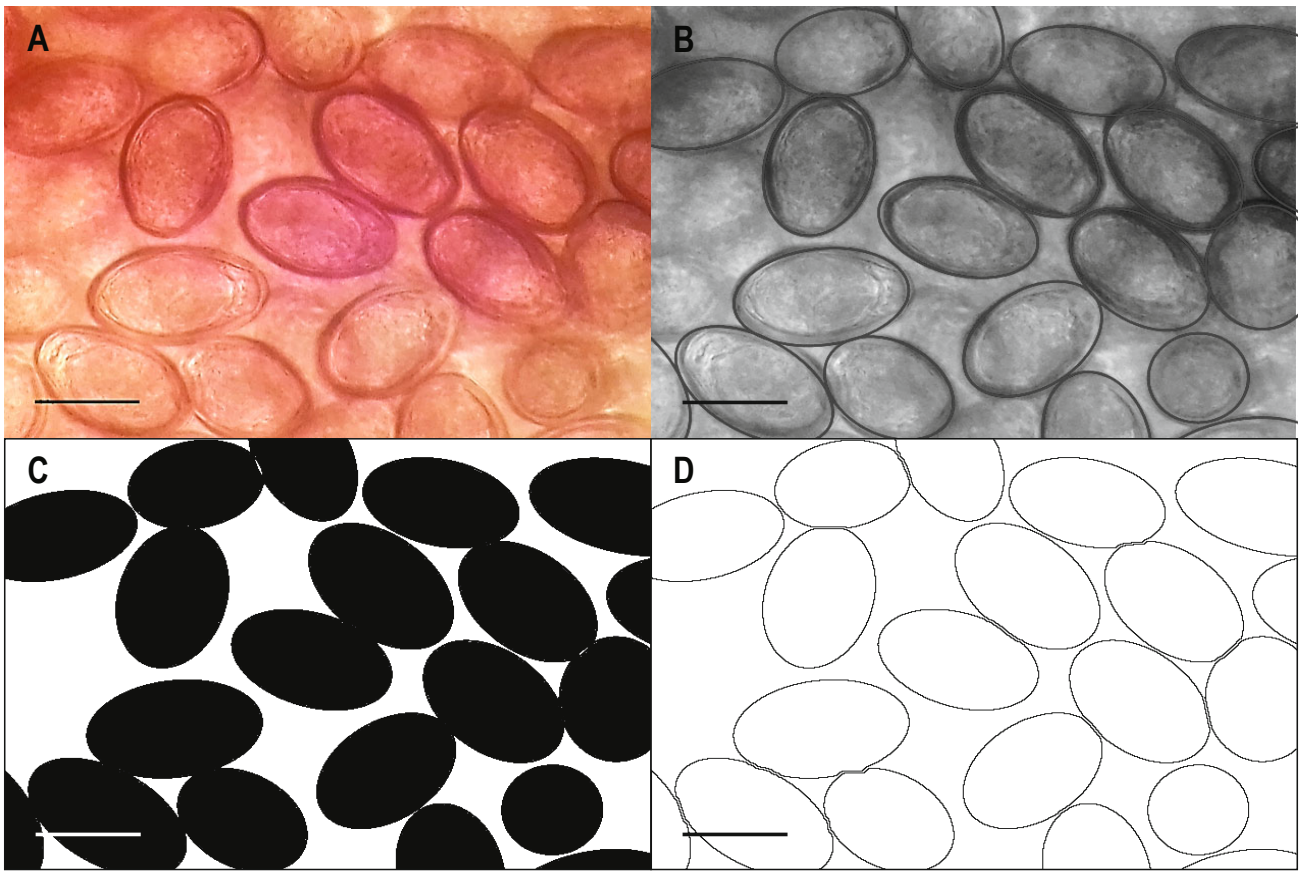

Fig. 2. Automatic counting process of *Eurytrema coelomaticum* eggs. (A) Original image. A micrograph (bright field) of the surface of *E. coelomaticum* eggs. (B) recognition pattern for structures by ImageJ software. It is the first step to the binarization process. (C) a secondary binary image is created. (D) transformation of the binary image to segmented lines with the capacity to measure the total area and perimeter of eggs. Scale bar 30 µm.

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The pancreas were washed inside of a bowl, so that, *E. coelomaticum* specimens could be recovered after the passage of water retained in the bowl through nylon sieves with a mesh of 150 µm. Subsequently, transverse and longitudinal sections were performed in the bovine pancreas in order to investigate the presence or absence of the parasites. For the light microscopy analysis the specimens were washed in 0.9 % physiological solution and fixed in AFA (Glacial Acetic Acid Solution, 2 parts, 37 % Formalin, 5 parts and 70 % Ethanol, 93 parts) (Corrêa *et al*., 2016). After fixation were placed between two glass slides and were lightly pressed for maintenance of their original shape. They were then stained with alcoholic chlorhydric carmine and observed under a light microscope (Olympus CX22 Led), coupled with a camera Moticam 5.0 MP and using Images Plus 2.0 software (Motic China Group Co LTD). All images used for comparison were photographed at the same magnification and with the same resolution (amount of pixels in the images)

To measure the total area of the adult animal, uterus and eggs, ImageJ software (NIH, USA) was used. In order to separate the uterus from the animal in the images was used a tool of creation of vectors in the Gimp 2.8 software (GNU Image Manipulation Program Open Source), and in this way it was possible to calculate the size of the organ through of creation of a secondary image with the same amounts of pixels of the original image. In ImageJ program, all images captured through the microscope and measured with the Images Plus 2.0 software were converted to gray scale images. Subsequently, the threshold function was used to improve the contrast of the structures to be analyzed in relation to the background. After the use of the threshold function, if there was any residual "noise" in the image (material that was not being evaluated), it was removed using the Gimp 2.8 software. For the delimitation between one object and another, the watershed tool was used. The total area of parasite, uterus and eggs was measured using the tools: Measure or Analyze Particles. The process of binarization, measurement and counting of the items evaluated in this work can be visualized in Figs. 1, 2 and 3.

From these data it was possible to estimate the total parasite area, uterine size and number of eggs per mm², adapting the methodology used by Rosati (2015). In order to verify the minimal number

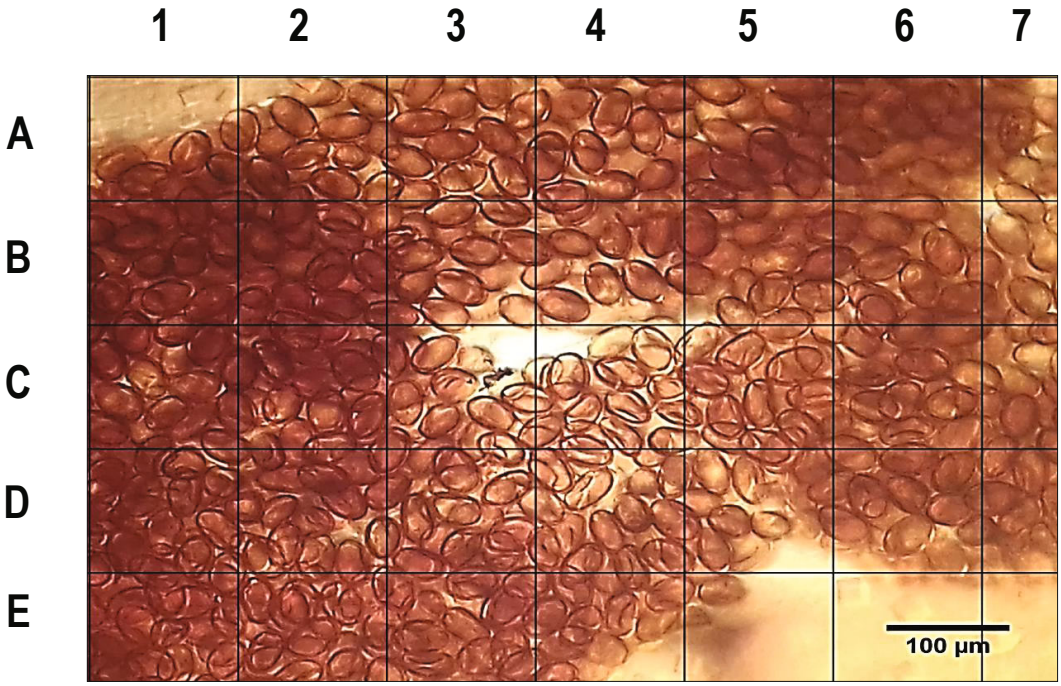

Fig. 3. Manual counting process: represent a picture with grids where was counted the *Eurytrema coelomaticum* eggs.

of eggs of *E. coelomaticum*, three scenarios were considered. In the first scenario, only the whole eggs observed in the field were counted. In the second process to estimate the number of eggs, were considered the areas of all visible structures and with clear delimitation. Manual count of eggs was also performed so that to establish the accuracy of the software. The comparison between the counting performed by the software and manually was performed only in this scenario because they are measures that have the closest characteristics and could be correlated. Finally, in the last scenario, it was considered an occupation of 100 % of the eggs in the image, since that the eggs into the uterus has three-dimensional characteristics, there are overlapping eggs and therefore cannot be correctly visualized and delimited in the image, which has a character two-dimensional. In this way it was calculated the maximum amount of eggs that can be seen in an image with 230 x 170 µm, taking as reference the average area of the eggs.

To measure the mean area of adult individuals, their respective uterus and the average egg size, an arithmetic mean was calculated based on 10 images. The manual counts were performed on images with the same area (in micrometers) and the values were extrapolated to mm², using a cross multiplication. The area used for counting the eggs by ImageJ software was 230 x 170 µm (totaling 0.0391 mm²) and the images used for manual counting had an area of 790 x 593 µm (equal to 0,468 mm²). To facilitate the manual counting of eggs, the selected images were divided into grids and these were numbered from 1 to 7 in the columns and from "A" to "E" in the lines according to Fig. 3.

Statistical Analysis

The paired T-test was applied to compare the number of eggs observed through manual counting and software. Linear regressions were made between the uterus area and total body area, and between the number of eggs observed by manual and software counts. The significance level of 5 % was used for both cases.

Ethical Approval and/or Informed Consent

All applicable national and institutional guidelines for the care and use of animals were followed. The study entailed samples of the normal meat inspection process of animals sent to slaughter for human consumption. This study was performed in strict accordance with the recommendations of the brazilian law nº 11.794/08 (which regulates procedures for the scientific use of animals) and Normative Resolutions of CONCEA (National Council for Control of Animal Experimentation).

Results

The adult specimens showed a flat body, fusiform or oval shape with an average length of 7.67 mm (± 0.34) and 3.66 (± 0.41) of width. The average area of adult individuals corresponded to 29.4 ± 4.26 mm², while the average area of the uterus represented 15.5 ± 3.18 mm². The total area of *E. coelomaticum* varied between 21 and 36 mm² and the total area of the organ oscillated between 9.5 and 20.6 mm². The eggs showed average values of area equal 980 µm² and average perimeter it was of 119 µm (n = 200). The

Table 1. Registered values for the total area of *Eurytrema. coelomaticum,* uterus size, proportional percent of the organ in relation to total area of parasite and eggs occupational rate into uterus lumen.

Sample	Total area (mm²)		Uterus/Animal	Occupancy rate
	E. coelomaticum	**Uterus (mm²)**	**(%)**	**(%)**
1	27.3	14.3	51.98	85
2	29.2	14.2	47.98	55.6
3	30.8	16.9	55.00	57
4	36	18.3	50.00	63
5	21	9.5	45.00	55.4
6	28.4	18.2	63.98	65.75
7	32.2	14.5	45.00	66.14
8	28	15.4	55.00	55.4
9	26.8	12.7	45.00	55
10	34.4	20.6	60.00	55.6
Average	29.4	15.5	51.9	61.38
Standard deviation	4.26	3.18	6.60	9.43
Minimum value	21	9.5	45.0	55
Maximum value	36	20.6	64.0	85

recorded values corresponding to the animals, uterus, proportion that organ represents in relation to the total size of the parasite and the percentage of occupation of the eggs in the lumen of the uterus are presented in Table 1.

The linear regression equation for the size of the uterus according to the total area of the animal (mm²) calculated was y = 0.6196x - 2.7614 and R^2 = 0.6875 which was significant (p <0.05). The prediction of the size of the uterus according to the body area of the parasite can be seen in Fig. 4.

In the first scenario, an average of 15 whole eggs was recorded (raging from 10 to 29 eggs per image). Considering only the average eggs seen in the images (15 eggs in 0.0391 mm²) and using a cross multiplication, the estimated number of eggs was equal 383.63 per mm². Using the estimation of whole eggs visible per mm² and considering the average area of the uterus of *E. coelomaticum*, it was estimated that an adult specimen has around 5,946 eggs in its uterus.

In second scenario, the average area occupied by eggs in lumen

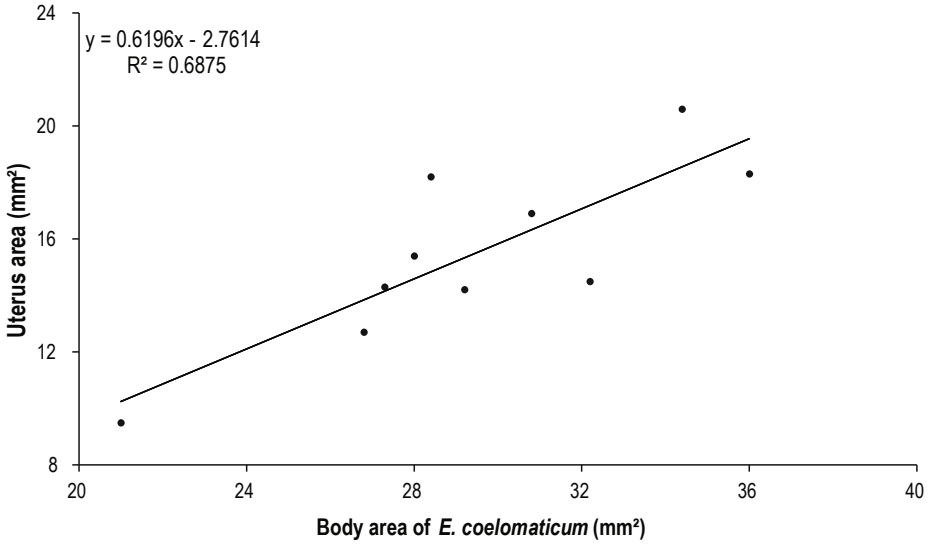

Fig. 4. Correlation between *Eurytrema coelomaticum* total area (mm²) and uterus size. Linear regressions equations were determined: y = 0,6196x - 2,7614 where y is estimated size of the organ and x is the body size (total area). R^2 = 0,6875, p< 0,05. The X axis it was changed, initializing in 20.

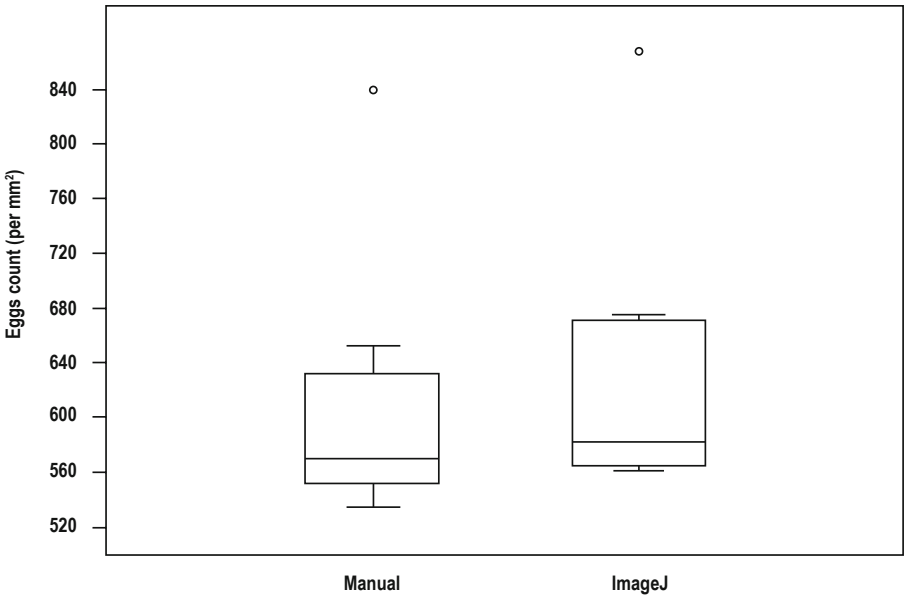

Fig. 5. Box plot of *Eurytrema coelomaticum* eggs per area (mm²) counted manually and automatized way. No significant difference were observed between counts with p value > 0.05.

of uterus was considered, i.e, whole eggs and portions that could be delimited and that corresponding to eggs. The area in image filled with eggs represented an average of 0.024 mm². The average percentage of the area occupied by eggs in the lumen of the uterus was 61.38 % (ranging from 55 % to 85 %). Multiplying the average percentage of eggs occupancy in the uterine lumen by the average area of the organ (15.5 mm²) and dividing the result by 100, reached the number of 9.51 mm² (corresponding to the area really used by eggs). Considering that an egg has an average area of 980 μm², in order to obtain the second estimate it was divided the area of occupation of the eggs (9.51 mm²) by average area of an egg, resulting at the estimated number of 9,704 eggs. Dividing the estimated number of eggs by the average area of the organ (15.5mm²) reached the value of 626 eggs per mm². The mean number of eggs counted manually was 9,366, with 604 eggs per mm² in the average. The distributions of the number of eggs and the respective outliers can be observed in Fig. 5.

In the T-test there was no significant difference considering an

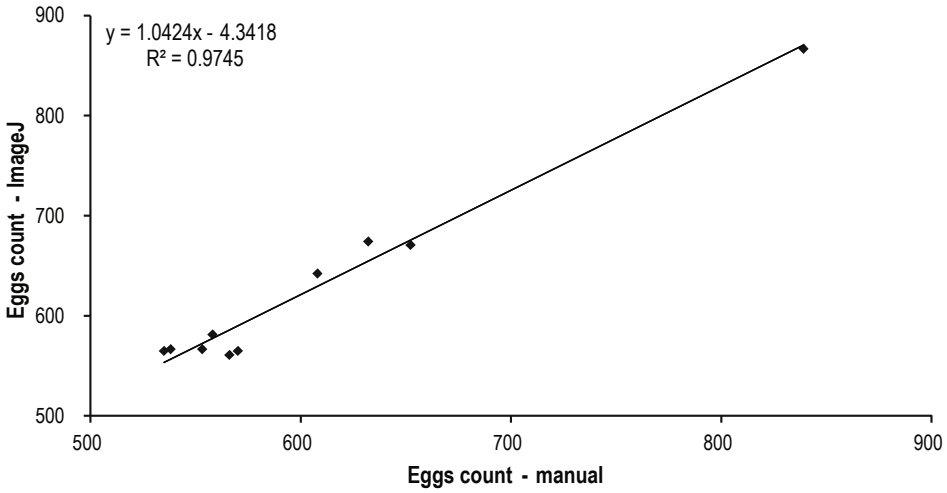

Fig. 6. Estimated amount of *Eurytrema coelomaticum* eggs in the parasite uterus, from cattle slaughtered in southern Minas Gerais, Brazil. Correlation between the number of eggs counted manually and through ImageJ software. Linear regression y = 1.0424x - 4.3418, where y is the predicted number of eggs estimated by the software and x the variable manual count; $R^2 = 0.9745$, $p < 0.05$. The X and Y axes it was changed, initializing in 500.

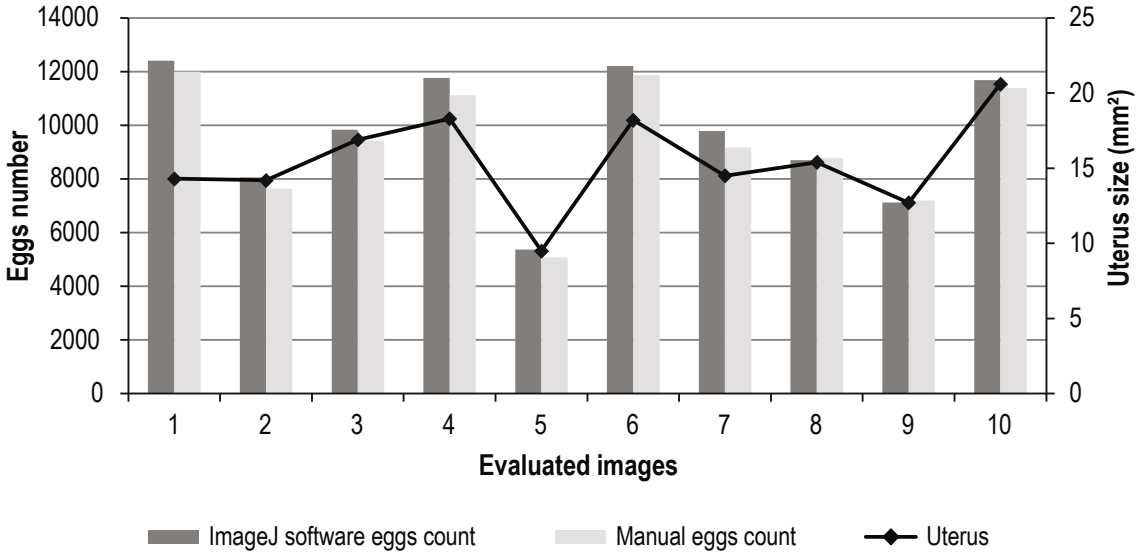

Fig. 7. Comparison of the number of manually counted eggs and the number of *Eurytrema coelomaticum* eggs registered by ImageJ software according to the area occupied by the eggs in lumen of the uterus and according to the size of the organ in square millimeters.

equivalent distribution for the variances of eggs numbers counted manually and estimated by the ImageJ software, with $p > 0.05$ ($p = 0.756$). A linear regression with a prediction interval of 95 % was performed for the number of eggs counted manually and automatic way, as can be seen in Fig. 6, with $R^2 = 0.9745$ and $y = 1.0424x - 4.3418$. The variation in the number of eggs is directly related to the size of the uterus as well as the occupancy rate in lumen by the eggs, as can be observed in Fig. 7.

Finally, in the third scenario, an occupancy rate of 100 % of the image per eggs was considered. Therefore, it was estimated that in 0.0391 mm² exist 39.89 eggs (0.0391 mm² ÷ 980 µm²) and consequently we reached the value of 1,020.20 eggs per mm². So, if consider the average size of the uterus of an adult animal it possible concludes that have the number estimate of 15,813. eggs in a *E. coelomaticum* specimen. A linear regression was performed and an equation was defined to estimate the number of eggs present in adults according to the area of the uterus. The equation for calculating the number of eggs considering an occupancy rate of 100 % of the uterus is: $y = 1020{,}4x + 1{,}82^{-12}$, where y is the predicted number of eggs and x the variable size of uterus in mm²; $R^2 = 1$, p value <0.05).

Discussion

According to Neuhaus (1978), the length of the uterus of some trematodes correlates with the size of the body. In the present study, it was observed that there is a relation between the size of the animal and the size of the uterus with p-value <0.05. The positive and significant relationship between the body size and uterus indicates that the addition of one is accompanied by proportional increase of another, without, however, requiring dependence between them, but allowing that from the first one can be estimated the value of the second.

The *E. coelomaticum* uterus has eggs clusters in its lumen, and there is no effective method to quantify the total number of eggs inside the organ without using methods that destroy internal structures of the parasite, causing losses of materials throughout the process of incision and removal of organ, affecting the count and later the results. ImageJ software provides precise measurements of surface area and is, therefore, an exceptional tool to support in counting and consequently creating estimates closer to reality. Mains *et al*. (2008) used successfully a digital image analysis supported with ImageJ program to estimate the number of aedine mosquitoes eggs using similar methodologies applied in this work. In the present study, a simple linear correlation was performed to evaluate the expected size of the uterus of adult animals using the total area of *E. coelomaticum* like parameter to calculate. This procedure was performed since the masses of the specimens were not verified, nor of organs in question, difficulting to estimate by means of an exponential logarithmic equation through of static allometric development. Valero *et al*. (2005) used as reference to evaluate the development of the uterus of *Fasciola hepatica* obtained in cattle from Bolivia, Spain and France an allometric growth model, where the curve shows an exponential growth. Neuhaus (1978) used to calculate the equation of the uterus length of *Dicrocoelium dendriticum, Pleurogenoides medians* and *Fasciola hepatica* the corrected body size that is equal to $\sqrt{\text{width} \times \text{length}}$. The author observed a sigmoid curve with the middle part presenting a linear section, that he considered an allometric growth.

In the literature, there is no description of estimates of total num-

ber of eggs present in the uterus of *E. coelomaticum*. There are studies that evaluated the oviposture rate of the parasite *in vitro*, and these studies observed a deposition ranging from 194 to 746 eggs per day in different physiological solutions (Yamamura, 1989; Brandolini & Amato, 2001). According to Bassani *et al*. (2007) the proportion of eggs eliminated and observed via EPG is not directly related to number of parasites in the host, even considering those who lay their eggs directly in lumen of intestine.

Our results indicate that simple linear regressions can be established to calculate the burden of eggs based in uterine size, and the equations can be defined by considering the occupancy rate in lumen of uterus or by considering a percentage of 100 %. Rosati *et al*. (2015), used a different design to estimate the number of eggs of blow flies, since they had masses of eggs (aggregates) and, therefore, it was possible to determine the depth of these agglomerates and thus to calculate the volume present in the analyzed samples by created a three-dimensional model to estimate the number of eggs through volume and not only the surface area. Despite the relative success with regard to egg estimation, it is important to note that the methodology adopted in this work has limitations such as the fact that the images analyzed by the software have two-dimensional characteristics, whereas the parasites have three-dimensional conformation. In this way the total number of eggs ends up being underestimated, being calculated in a certain way the minimum number of eggs that can be visualized, since those eggs that are not captured by the image end up not going into calculation of estimate. Therefore, considering the three scenarios established in the present work, it is evident that the minimum numbers of eggs present in *E. coelomaticum* uterus ranges from 5,946. to 15,813, and that is necessary to develop a methodology for extraction of the organ without loss of material and thus accurately determine the total number of eggs present.

Conclusions

The procedure performed in this study have some advantages like the fact that free software are used (ImageJ and Gimp), and these do not require expensive or specialized hardware. Another positive aspect related to technique is that the scanned images provide permanent records that can be checked by manual counting if this is desired.

Using the technique described in this work it is possible to estimate thousands of eggs in a short time and with a great accuracy. The time required for manual counting can be a complication in experiments that require egg counting, demanding a substantial effort, loss of time and resources. Therefore the method described in this work includes a substantial reduction of time, greater consistency in the obtained data, and decrease of errors besides taking the human bias. This study describes an important tool to quantify the number of eggs in a non-destructive way and aggregates information not explained previously by others works.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Respiratory and cardiopulmonary nematode species of foxes and jackals in Serbia

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Summary

As part of routine monitoring of foxes (*Vulpes vulpes*) and jackals (*Canis aureus*) on the territory of Vojvodina province (northern Serbia), an analysis of respiratory and cardiopulmonary parasitic nematodes was conducted. Both host species harbored *Eucoleus aerophilus*, *E. boehmi* and *Crenosoma vulpis*, whereas *Angiostrongylus vasorum* was found only in foxes. A high prevalence of infection (72.6 %) was noted for *E. aerophilus* in foxes. The remaining parasite species occurred less frequently in both host species. In all species where it could be quantified, a high degree of parasite aggregation within host individuals was noted. Single species infections were most common, whereas two and three species infections occurred less frequently in both host species. The distribution of abundance of *E. aerophilus* was affected by host sex, with abundances higher in male foxes. Sampling site and year influenced abundance variation in *E. boehmi*.
Keywords: respiratory nematodes; cardiopulmonary nematodes; red fox; jackal; Serbia

Introduction

Red foxes (*Vulpes vulpes*) are principal reservoirs of numerous parasites, owing to their wide ranging geographic distribution (Duscher *et al*., 2014), frequent urbanization (Contesse *et al*., 2004; Morgan *et al*., 2008; Millán *et al*., 2014) and population increase following oral vaccination programs throughout Europe (Goszczinski *et al*., 2008; Borecka *et al*., 2009). Among these parasites are respiratory nematodes such as *Eucoleus aerophilus* (Creplin, 1839), *E. boehmi* (Supperer, 1953) and *Crenosoma vulpis* (Dujardin, 1844) and the cardiopulmonary nematode *Angiostrongylus vasorum* (Baillet, 1866) (Traversa *et al*., 2010; Otranto *et al*., 2015; Latrofa *et al*., 2015). Another canine species that plays a significant part in dissemination of pathogens is the golden jackal (*Canis aureus*), whose populations are also on the rise, particularly on the Balkan Peninsula (Šálek *et al*., 2015).

Eucoleus aerophilus (Enoplida, Capillaridae) lives submerged in the epithelium of the trachea, principal bronchi and the bronchi of the caudal lobes (Traversa *et al*., 2009), rarely localizing in the bronchiolar epithelium of infected animals (Nevárez *et al*., 2005). Its life cycle is described as either direct or indirect with earthworms as paratenic hosts (Di Cesare *et al*., 2012, 2014; Veronesi *et al*., 2013). While its primary hosts are various carnivorous mammals, it has also been known to infect humans. Twelve cases of human *E. aerophilus* infection have been reported worldwide, including one in Serbia, where a woman admitted with clinical signs pointing to bronchial carcinoma was found to be infected with the parasite (Lalošević *et al*., 2008).

The congeneric species *E. boehmi* has been increasingly reported in many European regions since the turn of the century (Sréter *et al*., 2003; Lalošević *et al*., 2013; Veronesi *et al*., 2014). This parasite inhabits the nasal cavity and frontal sinuses of the host,

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and its life cycle is poorly known. The hosts exhibit clinical manifestations such as sneezing and increased nasal secretion, or subclinical signs of respiratory obstruction. More recently, the parasite has been considered a main cause of intracranial disease, such as meningococcal encephalitis in dogs (Clark *et al*., 2013). However, in spite of its pathogenicity and occurrence in several European countries (Veronesi *et al*., 2014; Otranto *et al*., 2015; Hodžić *et al*., 2016a), *E. boehmi* remains underestimated and is often overlooked. Present knowledge of its range in domestic and wild animals in Europe is scarce, mostly available from studies that focus on other cardiopulmonary parasites (Conboy, 2009; Hodžić *et al*., 2016a).

Adults of *Crenosoma vulpis* (Strongylida, Crenosomatidae) can be found in small bronchi and bronchioles of all pulmonary lobes in wild and domestic canids (Bihr & Conboy, 1999). Its intermediate hosts are mollusks, and the distribution of *C. vulpis* larvae is directly influenced by climatic factors such as temperature and humidity, as well as occurrence of suitable hosts. The nematode, much like *E. aerophilus*, causes damage to the pulmonary parenchyma and chronic respiratory bronchitis. Where infections are particularly intense, bronchopneumonia may cause death of the host (Holmes & Kelly, 1973; Bowman *et al*., 2002; Taylor *et al*., 2007).

Angiostrongylus vasorum (Strongylida, Angiostrongylidae) is a highly pathogenic metastrongylid nematode that parasitizes on different carnivore species. L1 stages are localized in the lungs, with adults found in the pulmonary artery and right side of the heart. Gastropods act as intermediate hosts and L3 carriers (Woolsey *et al*., 2017). Frogs and birds may also figure into its life cycle as intermediate or paratenic hosts (Bolt *et al*., 1993; Mozzer & Lima, 2015). Dogs infected with this nematode display subclinical, sometimes even fatal cardiopulmonary and neurological symptoms (Barutzki & Schaper, 2009; Moeremans *et al*., 2011). Its endemic hotspots are in Western Europe (France, southern Great Britain, Ireland). In Central and Eastern Europe, *A. vasorum* was reported from dogs and foxes in Croatia (Rajković-Janje *et al*., 2002), Greece (Papazahariadou *et al*., 2007), Hungary (Majoros *et al*., 2010), Poland (Schnyder *et al*., 2013), Romania (Deak *et al*., 2017) and Slovakia (Hurníková *et al*., 2013), with a tendency of spreading further southeast. In Vojvodina, the northern province of Serbia, there has been only one documented case of canine *A. vasorum* infection (Simin *et al*., 2014).

Previous studies of respiratory and cardiopulmonary nematodes have shown that these parasites have high pathogenic potential. Thus, it is necessary to develop efficient measures of parasite control and diagnosis, and to monitor their transmission pathways in domestic and wild animals. The aim of this study was to bring attention to the presence of these parasites in wild carnivores – foxes and jackals – of the Vojvodina region in Serbia, and to emphasize the epidemiological and epizootiological significance of carnivores as hosts of respiratory nematodes that cause diseases harmful not only to wild and domestic animals, but also to man.

Materials and Methods

Sample collection and processing

Adult foxes and jackals were collected from 2009 to 2016 as part of routine rabies diagnostic procedure in the Pasteur Institute of Novi Sad, National Reference Laboratory for rabies. The animals were collected from various regions of Vojvodina, obtained through cooperation with local hunting clubs. Both foxes and jackals were sampled in roughly the same time period, the majority of animals captured from autumn to early spring (October to March). Serbian law lists the red fox and the jackal as protected species. However, their status and protection regime are regulated by hunting legislation, and hunting season lasts throughout the year for both species. The animals were sexed, and their body mass (kg) and total body length with tail (cm) were measured. Tracheas and lungs of all red foxes and jackals were carefully cut open with scissors and examined visually for the presence of respiratory parasites.

Samples of the trachea and lungs were collected from each animal, in order to test the bronchial and bronchiolar contents for the presence of *E. aerophilus* adults and eggs and *C. vulpis* adults and larvae. Tracheal samples were taken from the larynx to the tracheal bifurcation, together with the lungs. The tracheas were cut open along the frontal side with scissors, and then examined under a stereomicroscope together with the bronchi and bronchioles of the lungs. Recovered *E. aerophilus* adults and bronchial contents were immediately prepared in 50 % glycerol. Slides of the capillarid nematodes were then examined under an optic microscope, magnified 40 to 100 times. Adult stages of nematodes recovered from trachea and bronchi of the foxes were identified at the species level using morphological keys.

Samples of the nasal mucosa obtained by scarification of the nasal cavity from wild animals were tested for the presence of *E. boehmi*. After extraction, the tissue was placed in 0.9 % saline and immediately examined under stereomicroscope. *Eucoleus boehmi* adults were prepared in 50 % glycerol and examined under an optic microscope in the same way as for the previous species.

Data analysis

Examinations for the presence of *E. aerophilus* in the respiratory tract were carried out starting from 2009 and 2014 for foxes and jackals respectively. Infection data for this nematode were obtained from 351 foxes and 49 jackals, and these were the host individuals that formed the sample for quantitative analyses of infection. Examination for the remaining two species, *E. boehmi* and *C. vulpis* was conducted starting from 2013 for foxes, and 2014 for jackals. Data on quantitative parameters of infection for *E. boehmi* are based on 184 foxes and 30 jackals. The sample for *C. vulpis* consisted of 205 foxes and 49 jackals. *Angiostrongylus vasorum* was found sparingly; since nematode specimens were not precisely quantified and the heart was not examined, no further quantitative analysis was performed for this parasite species, nor was it considered in co-infection analysis.

Quantitative parameters of infection are stated according to Bush *et al*. (1997). Data on prevalence, mean and median infection intensity, mean abundance and dispersion index are given for *E. aerophilus* and *E. boehmi*. The dispersion index was used as a measure of parasite aggregation within hosts (Shaw & Dobson, 1995). For *C. vulpis*, large numbers of larvae made precise counts of individuals difficult to obtain, and thus only prevalence is given for this nematode species. Prevalence values of *E. aerophilus*, *E. boehmi* and *C. vulpis* were compared between foxes and jackals via the exact unconditional test (Reiczigel *et al*., 2008). Differences in mean intensities and mean abundances of *E. aerophilus* and *E. boehmi* between the two host species were tested with a bootstrap test with 20000 replications. All quantitative analyses and statistical tests were performed in Quantitative Parasitology 3.0 software (Rósza *et al*., 2000). To calculate the percentage of animals infected with one, two or three respiratory parasite species, a subsample consisting of hosts with data for all three recurring nematodes (*E. aerophilus*, *E. boehmi* and *C. vulpis*) was created. This subsample consisted of 179 foxes and 30 jackals. Based on this data, percentages of foxes and jackals carrying specific single or combined infections were determined.

The fox sample size and high prevalence values for the two *Eucoleus* species enabled an analysis of factors influencing parasite abundance. The selected factors were year of sampling, site of sampling (the township from which the host individual originated), total length (body and tail), body mass and sex. The sample was formed exclusively from host animals for which all afore mentioned data were available. This resulted in 210 foxes for *E. aerophilus*, and 150 for *E. boehmi*.

In order to determine which of the listed factors exerted influence on nematode abundance, the abundance data was fitted to a Generalized Linear Model (GLM). The initial model contained all of the factors and their two-way interactions as terms predicting the numerical response. The factors and interactions that failed to pass the significance threshold were eliminated stepwise until the most satisfactory minimal model was obtained. GLM analysis was performed in R statistical software (R core team, 2013), utilizing its standard packages.

Ethical Approval and/or Informed Consent

The animals used in this study were captured and obtained as part of routine rabies diagnosis procedure, according to a program set up by the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (Official Gazette of the Republic of Serbia Nos. 43/17 and 11/18). The research related to animals has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

Results

The prevalence of *E. aerophilus* was 72.6 % in foxes, nearly double the value it had in the jackal sample. In addition, infected foxes typically carried more nematode individuals than jackals, and mean abundance of the parasite was also higher in foxes. Both values of the dispersion index d were greater than 1, signifying an aggregated distribution: the majority of the nematodes were found in a small number of hosts. The differences in prevalence, mean intensity and mean abundance of *E. aerophilus* between foxes and jackals were all statistically significant at $p<0.0001$ (Table 1).

Prevalence of *E. boehmi* was, as in the previous species, higher in foxes than in jackals, but this difference was far more subtle than for *E. aerophilus*. Infected jackals carried more parasites on average than infected foxes, but mean abundance was higher in the fox sample. Dispersion index values point to parasite aggregation as in *E. aerophilus*, particularly evident in the jackal sample: all 6 nematodes were found in 3 host individuals, with the remainder of the jackals uninfected. The mean intensity of *E. boehmi* infection in jackals was significantly higher than in foxes ($p=0.022$). The differences in prevalence ($p=0.357$) and mean abundance ($p=0.595$) of the parasite between the two host species were not significant (Table 2).

Just over 6 percent (6.1 %) of the 49 examined jackals carried *C. vulpis*. While no adult worms were found, large numbers of larvae served as evidence of presence. A higher prevalence value of 16.6 % was found in foxes, but this difference was not statistically significant ($p=0.088$). Since precise counts of parasite individuals are unavailable, other quantitative measures were not calculated. However, while no dispersion index is given, it should be noted that all 44 adult worms were found in 14 of the 205 analyzed foxes. Both foxes and jackals displayed the same pattern of parasite community richness. Infections with a single nematode species were the most common in both hosts, with *E. aerophilus* infecting the largest number of individuals, followed by *E. boehmi* and *C. vulpis* in that order. Infections with two nematode species were

Table 1. Quantitative parameters of infection with the nematode *E. aerophilus* in foxes and jackals in Vojvodina, Serbia.

Host	Number of individuals	P%	MI	MedI	MA	d
V. vulpes	351	72.6 (67.7 – 77.1)*	8.7 (7.4 – 10.2)*	4	6.3 (5.3 – 7.4)*	17.3
C. aureus	49	32.7 (20.6 – 46.9)	1.8 (1.3 – 2.5)	1	0.6 (0.3 – 0.9)	2

P% – prevalence; MI – mean intensity; MedI – median intensity; MA – mean abundance; d – dispersion index; 95 % confidence intervals given in parentheses where applicable. Asterisks denote statistically significant differences.

Table 2. Quantitative parameters of infection with the nematode *E. boehmi* in foxes and jackals in Vojvodina, Serbia.

Host	Number of individuals	P%	MI	MedI	MA	d
V. vulpes	184	17.9 (13 – 24.1)	1.48 (1.2 – 1.9)	1	0.3 (0.1 – 0.4)	1.8
C. aureus	30	10 (2.8 – 26.3)	2*	2	0.2 (0 – 0.4)	1.9

P% – prevalence; MI – mean intensity; MedI – median intensity; MA – mean abundance; d – dispersion index; 95% confidence intervals given in parentheses where applicable. Asterisks denote statistically significant differences.

less frequent, and neither foxes nor jackals were infected with the combination *E. boehmi-C. vulpis*. Infections with all three species were rare in foxes, and altogether absent in jackals (Table 3).

The only factor that significantly influenced the variation in abundance of *E. aerophilus* in red foxes was host sex (p=0.0005). Mean abundance of the parasite was greater in males (6.22) than in females (2.47) of this host species. Sampling site (p<0.0001) and year (p=0.0002) influenced abundance of *E. boehmi* in the same host, as well as the interactions year:site (p=0.0454), year:body mass (p=0.0012) and site:total length (p=0.0033). Body mass (p=0.4301) and total body length (p=0.9716) did not influence the abundance of *C. boehmi* in foxes as independent factors. Furthermore, due to the disproportional representation of different sample categories, the fact that sampling site and year play a part in determining the abundance variation in *E. boehmi* carries little informative value. The years with a significantly higher abundance of the parasite (2013 and 2014) are also the years with the smallest number of host animals analyzed, increasing the values of the parameter. The township of Zrenjanin, which had a significantly higher *E. boehmi* abundance compared to others, contributed only 3 foxes to the total sample, skewing the result.

Aside from the three respiratory nematodes listed above, the cardiopulmonary parasite *Angiostrongylus vasorum* was also found in foxes only. Three host individuals were infected with three worms in total, resulting in a prevalence value of 1.8 %.

Discussion

Eucoleus aerophilus prevalence in Vojvodina was 72.6 % and 32.7 % in foxes and jackals respectively. It should be noted that previous studies report disparities in prevalence values obtained via necropsy and those estimated by coprological examination. The former method is often described as the golden standard for confirming respiratory nematode infection, with a much higher sensitivity than analyses of fecal matter (Magi *et al*., 2009, 2015). The prevalence of *E. aerophilus* reported herein is relatively high when compared with infection percentages from other European countries. For example, Magi *et al*. (2015), in addition to their own results, summarize data from 12 other studies from different parts of Europe, only 4 of which report prevalence in foxes higher than 72.6 %. Lalošević *et al*. (2012), in an earlier paper concerned with the Vojvodina province of Serbia, state a prevalence of 84 %, a value far closer to the one found in the present study. On the other hand, Ilić *et al*. (2016) find a prevalence of 23.7 % in foxes, and an absence of infection in jackals. Their sample, however, consisted of animals originating south of the Sava and Danube rivers, outside of the Vojvodina region which, as data suggests, provides an ideal environment for the transmission and sustained presence of this nematode species. According to Lalošević *et al*. (2012), a higher rate of parasite transmission in Vojvodina is caused by the humid conditions of the southern part of the Pannonian basin, a consequence of an abundance of canals and tributaries between the major rivers. Tolnai *et al*. (2015) reach a similar conclusion in their analysis of climatic factors shaping the transmission of respiratory and cardiopulmonary nematodes in Hungary, which is also part of the Pannonian basin. They find that increasing humidity and decreasing average annual temperature create conditions suitable for the spreading of *E. aerophilus*, primarily due to the important role of water in the survival of the eggs which are susceptible to desiccation. The same authors report that the spatial distribution of this nematode was less localized than that of the other two species in the study (*C. vulpis* and *A. vasorum*), indicating that the proposed direct life cycle of *E. aerophilus* makes it less sensitive to the effects of climatic factors.

The sex of host foxes influenced the abundance of *E. aerophilus*, with males being more infected. Morgan *et al*. (2008) also note a larger presence of this nematode in males, but other studies did not find a connection between host sex and quantitative infection

Table 3. Percentage of host individuals infected with one (*E. aerophilus*, *E. boehmi* or *C. vulpis*), two (one of three specific combinations) or three respiratory nematode species.

Host species	Single species infections			Two species infections			Three species infections
	Ea	*Eb*	*Cv*	*Ea+Eb*	*Ea+Cv*	*Eb+Cv*	*Ea+Eb+Cv*
V. vulpes	58.2 %	3.7 %	1.5 %	14.9 %	17.9 %	0 %	3.7 %
C. aureus	66.7 %	16.7 %	0 %	8.3 %	8.3 %	0 %	0 %

Ea – Eucoleus aerophilus; *Eb – Eucoleus boehmi*; *Cv – Crenosoma vulpis*

parameters (Saeed *et al*., 2006; Magi *et al*., 2015). The effect of host sex on parasitic burden remains a highly complex aspect of parasitological studies, with various authors reaching divergent conclusions. Nevertheless, in cases where males have more pronounced infections, an explanation based on the differential effect of natural selection on the sexes is most commonly proposed: the immunosuppressive properties of male sexual hormones and certain types of sex-specific behavior make males more likely to be infected (Zuk & McKean, 1996). Age, season and population density of the host (Saeed *et al*., 2006; Lalošević *et al*., 2012) are other factors reported as having significant roles in determining infection characteristics of *E. aerophilus*, but they were not taken into account in the present study. Additionally, results may vary depending on the author: Morgan *et al*. (2008), for example, did not find a seasonal variation in infection probability. Teasing apart the factors that determine the distribution and infective capacity of this respiratory nematode is an important task, as it allows us to make educated guesses on the patterns of its spreading and enables preventive measures. The parasite causes visible pathological changes in its hosts (Nevárez *et al*., 2005). Considered together with its complicated diagnosis, zoonotic potential and expansion into non-endemic areas, this makes it a sizeable threat to pets – domestic dogs and cats (Traversa *et al*., 2010). In Denmark, Saeed *et al*. (2006) report significantly higher prevalence of capillarid nematodes in urban foxes, compared to rural specimens. It is these urban foxes that act as a source of disease for domestic animals and people (Magi *et al*., 2009). The twelve cases of human infection found in literature, including one from Serbia (Lalošević *et al*., 2012), further emphasize the importance of raising awareness of the parasite's presence and developing precise diagnostic techniques that help differentiate between clinical signs that can easily be attributed to other respiratory conditions.

Single infections with *E. boehmi* and *C. vulpis* were far less frequent than those with *E. aerophilus*. In addition, whenever two species were found in fox and jackal hosts, the combination included *E. aerophilus*: the remaining two species were never found together, unless *E. aerophilus* was also present. This may point to a synergistic interaction, where *E. aerophilus*, by weakening or modulating the hosts' immune response, facilitates the establishment and survival of other respiratory parasites. Evidence for such synergistic interactions exists in other host-parasite systems, for example for the intestinal nematode *Heligmosomoides polygyrus* in rodents (Behnke *et al*., 2001, 2009; Maizels *et al*., 2004). Great caution needs to be taken when making assumptions such as these. Interactions and associations between parasite species remain poorly understood: experimental studies in strictly controlled conditions or analyses based on predetermined null models are necessary to determine whether any changes in abundance occur when two species coexist within the same host (Poulin, 2001), and these have yet to be carried out for respiratory nematodes. The present study focused on respiratory parasites only. It's certainly possible that the examined hosts carried other parasites (intestinal for example), and that they are in fact the ones driving the interactions. Parasites may even behave as independent units within their hosts, their distribution determined by natural selection narrowing them down to specific predilection spots that carry the best conditions for survival. If this is true, each species would occupy its own isolated niche and interspecific interactions would be nonexistent (Poulin, 2001). In this light, the findings of the present co-infection analysis could be interpreted quite differently. For example, it could simply be that *E. aerophilus* has greater longevity than the other two species, outliving them in the host and thus more often appearing in single and multiple infections.

Eucoleus boehmi was found in the nasal mucosa of 17.9 % of examined foxes and 10 % of examined jackals, with an aggregated distribution within host individuals. Studies of extraintestinal nematode parasites of foxes in Hungary (Sretér *et al*., 2003) and Norway (Davidson *et al*., 2006) also report this species, with prevalence values of 8 % and 51 % respectively. Recently, *E. boehmi* has been receiving more attention and research has intensified. Prevalence in foxes ranges from 30.7 % in Italy (Veronesi *et al*., 2014) to 71 % in Denmark (Al-Sabi *et al*., 2013), and even 83 % in Austria (Hodžić *et al*., 2016b). On the other hand, data on jackals are far more lacking. According to a thorough review of jackal parasites by Gherman & Mihalca (2017), *E. boehmi* has, to this date, only been found in jackals in Russia, with a prevalence of 30 %. Population expansion of foxes and jackals, together with relatively high prevalence of the parasite established by contemporary research, position these canids as natural reservoirs and disseminators of the nematode. This, consequently, mandates detailed studies of nasal eucoliosis in wild animals and better diagnostic measures for domestic animals.

Foxes in Vojvodina had a relatively low prevalence of *C. vulpis* (16.6 %). In jackals, based on larvae only, the percentage of infected hosts was 6.1 %. The life cycle of this parasite is indirect, with snails as intermediate hosts. Parasite prevalence varies in different European countries: 24 % in Hungary (Sréter *et al*., 2003), 24.9 % in Austria (Lassnig *et al*., 1998), 28.2 % in the United Kingdom (Willingham *et al*., 1996), 13-18 % in Spain (Miquel *et al*., 1994), 13 % in Vojvodina province of Serbia (Simin *et al*., 2012) and 4.5 % in the Netherlands (Borgsteede, 1984). Recent data show that this nematode is the leading cause of respiratory disease in domestic dogs in Spain, Portugal, Switzerland and Germany (Unterer *et al*., 2002; Madeira de Carvalho *et al*., 2009; Barutzki & Schaper, 2009). Such conclusions highlight the importance of monitoring the occurrence of *C. vulpis* in wild animals. Presence of the parasite in a specific area should be taken into account when dogs with symptoms of inflammatory diseases are encountered.

In the current study, the cardiopulmonary nematode *A. vasorum* was not found in jackals, in accordance to Takács *et al*. (2014). However, Gavrilović *et al*. (2017) and Simin *et al*. (2014) report on pneumonia caused by this parasite in jackals and foxes. We only found the nematode in three individual foxes (1.8 %), each one carrying a single worm. Such low prevalence values are report-

ed from neighbouring countries including Croatia, Hungary and Romania. These areas appear to be the easternmost limits of its distribution, with hotspots located in Western and Central Europe (Deak *et al*., 2017). A rise in infestation with this nematode species was noted in dogs in Italy (Traversa *et al*., 2008), Germany and Denmark (Taubert *et al*., 2009). Eleni *et al*. (2014) report the first occurrence of the so-called French heartworm in wolves in Italy, and Barutzki and Schaper (2009) document its rising infection trend in Germany and report on its distribution in the country. The spread of angiostrongyloidosis may occur via the transport of dogs from one country to another; indeed, it appears that the nematode was brought into Denmark via dogs originating in France. Since the disease is often fatal to domestic dogs, and its distribution is still under-documented, all researchers agree that *A. vasorum* requires greater attention as a threat to domestic and wild animal health. This is further supported by the results put forward by Gillis-Germitsch *et al*. (2017), stating that dogs seem to be developing tolerance to the parasite. This would enable prolonged survival for the heartworm and facilitate its spread, allowing it to reach new areas before being detected in its carriers. This could possibly explain new hotspots and epizootics in certain parts of Europe.

In conclusion, the present study found four species of respiratory and cardiopulmonary nematodes in foxes and jackals, two wild canids currently expanding their ranges in Europe due to their adaptability and resilience, coupled with the benefits of oral rabies vaccination. Domestic animals such as dogs and cats, as well as humans under certain circumstances, may become hosts of these parasites, the clinical consequences of these infections being far from negligible. An increase in international transport of animals and goods, with insufficient monitoring of higher levels of trade, is expected to result in a further expansion of *E. aerophilus* (Traversa *et al*., 2010), and with it other respiratory and cardiopulmonary nematodes, rendering studies such as this one indispensable in the near future. All of the above explicitly points to the significance of such research and the necessity for its intensification.

Conflict of Interest

Authors state no conflict of interest.

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New data on endohelminth communities of barbel *Barbus barbus* from the Bulgarian part of the River Danube

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Summary

Species diversity and composition of the parasite communities of barbel (*Barbus barbus*) at the infracommunity and component community levels were studied in the Lower Danube River, Bulgaria. During the two-year investigations, five parasite species have been found in 92 host fish: *Bathybothrium rectangulum* (Cestoda), *Acanthocephalus anguillae* and *Pomphorhynchus laevis* (Acanthocephala) and larval stages of *Contracaecum* sp. and *Raphidascaris acus* (Nematoda). *Bathybothrium rectangulum* and *R. acus* found in barbel represented new host records in Bulgaria. Parasite communities of barbel were species-poor and highly unbalanced. *Pomphorhynchus laevis* represented the dominant (core) species (prevalence 98.9 %), the second most frequent component parasite was *Contracaecum* sp. (P = 14.1 %) and remaining three species occurred only accidentally in barbels. Differences in species richness, prevalence, intensity of infection and ecological indices between individual seasons (spring, summer, autumn) were statistically significant, but considerably affected by unequal species structure of communities with highly prevailing *P. laevis*. Low parasite species diversity of barbel and low values of most ecological indices, when compared with previous studies in this area (or other Bulgarian parts of the River Danube) might indicate that environmental conditions are impaired and thus, not favourable for the development of barbel parasites (primarily to their intermediate host survival) in the Lower Danube River of Bulgaria.
Keywords: *Barbus barbus*; helminths; parasite community structure; seasonality; Danube River; Bulgaria

Introduction

The River Danube is a longest river of the Central and Eastern Europe, which flows 2,860 km through ten countries or touches their borders. Its stream use to be divided into three broad sections with the delta representing a separate and unique system. The Upper Basin extends from the source in Germany, to the Devín Gate (Austria/Slovakia border), the Middle Section to Iron Gate dams (the Serbia/Romania border), and the Lower Section to the entrance of the Delta, with the whole section of the Bulgarian river bank (for review see Tubic *et al*., 2013). The barbel, *Barbus barbus* (Linnaeus, 1758) is indigenous to the ichthyofauna of the Danube Basin. As the potamodromous and benthopelagic species, it feeds chiefly on benthic invertebrates such as small crustaceans, insect larvae, molluscs, mayflies and midge larvae, but also small fish and occasionally algae (Froese & Pauly, 2018). Parasite fauna of this fish reflects these food habits and local ecological conditions. The fish community in the shoreline zone of the Danube River

in Bulgaria was investigated by Polačik *et al.* (2008). In this study, they identified 44 fish species, barbel, represented rarely occurring and low abundant fish species.
Fish and parasite communities from the lower section of the Danube River, including the Bulgarian part of the river, were often studied (Margaritov, 1959, 1966; Kulalovskaya & Koval, 1973; Kakacheva-Avramova, 1977, 1983; Kakacheva *et al*., 1978; Nedeva *et al*., 2003; Polačik *et al*., 2008,; Atanasov, 2012; Kirin *et al*., 2013, 2014 etc.), but only Nachev & Sures (2009) and Nachev (2010) examined specifically barbel.
The aim of this paper was to find the diversity of endoparasites of barbel and evaluate the structure of their communities in the Bulgarian part of the Lower Danube River.

Materials and Methods

During spring, summer and autumn of 2015 and 2016, a total of 92 specimens of barbel were collected from the Lower Danube River near the village Vetren, Bulgaria (44°133'N, 27°033'E). The village is situated on the riverside, in the north-eastern part of the Danube Valley.
Barbels were caught using gill nets and by angling under a permit issues by the Ministry of Agriculture and Food of the Republic of Bulgaria. The scientific and common names of fishes are used according to the FishBase database (Froese & Pauly, 2018).
The fish were weighed (a mean weight was 445.9 ± 40.1 g and ranged between 220 – 788 g) and measured (mean standard body length 36.1 ± 1.0 mm, range 290 – 450 mm). Corresponding values for individual seasons are shown in the Table 1. The sample size can yield to reliable estimates the parasite abundance (Shvydka *et al*., 2018). The fish were immediately after their capture examined for gastrointestinal and tissue helmiths (an incomplete parasitological study) using standard techniques. Helminths were cleaned in a saline solution and fixed in 70 % ethanol. Cestodes were stained with acetic carmine and mounted as permanent slides in a Canada balsam according to Georgiev *et al.* (1986) and Scholz & Hanzelová (1998). Acanthocephalans and nematode larvae were examined as temporary slides in ethanol-glycerine and glycerine, respectively, and identified by use of keys by Petrochenko (1956), Bykhovskaya-Pavlovskaya (1985), Khalil *et al.* (1994) and Moravec (2013) and Lab Compound Microscope XS-213. In total, 6,408 adult or larval helminth specimens were collected (Table 2).
The helminth community structure was studied during three seasons at component community and infracommunity levels. Ecological terms were used according to Bush *et al*. (1997); a prevalence (P, %), mean abundance (MA) and mean intensity of infection (MI). The component data were determined by the total number of species, Shannon diversity index (H'), Pielou's evenness index (E) and Berger-Parker dominance index (d) according to Magurran (2004). The dominance of the component helminths within communities was determined according to the prevalence criterion (P) proposed by Kennedy (1993) as accidental (P<10), component (10<P<20) and core (P>20) species. The infracommunity data were calculated using the mean number of species, mean number of individual helminth specimens, Brillouin diversity index (HB), (Magurran, 2004; Kennedy, 1993, 1997). The quantitative similarity between parasite communities during three seasons was determined by the Sorensen index (Percentage similarity index, Ics), (Krebs, 1999).
The significance of seasonal changes in the prevalence and MI was evaluated by the Chi-square (χ^2) and Two-sample t-test, respectively. A one-way ANOVA was used to compare the mean number of helminth species and the Brillouin diversity index within infracommunities, same as mean number of *P. laevis* with mean number of specimens of all other species. All statistical tests were performed using statistical software programs Quantitative Parasitology, version 3, Rozsa *et al*. 2000; STATISTICA 6.0 program and Microsoft Excel/Windows® XP Home Edition.

Ethical Approval and/or Informed Consent

This research carried out on fish has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

Results

Helminth community structure of barbel
All barbels examined (92/92; P = 100 %) were infected and following parasite species were identified: the tapeworm *Bathybothrium rectangulum* (Bloch, 1782), thorny-headed worms *Acanthocephalus anguillae* (Müller, 1780) and *Pomphorhynchus laevis* (Zoega in Müller, 1776), and nematode larvae of *Contracaecum* sp. and *Raphidascaris acus* (Bloch, 1779). All but one are generalists, *B. rectangulum* is specific parasite to *Barbus* spp. (Protasova, 1977; Moravec *et al.*, 1997). Adult stages of the cestode *B. rectangulum* and acanthocephalans *A. anguillae* and *P. laevis* (all autogenic) occurred in a host intestine. Allogenic nematode larvae were found either encapsulated in various internal organs and an intestine serosa, or free in abdominal cavity of infected fish.

Infracommunities of barbel helminth endoparasites
Species richness in helminth infracommunity of barbel ranged from one to three parasite species. A total of 74 fish individuals were infected with a single helminth species (80.43 %); 16 barbels harboured two helminths (17.40 %); namely, 11 barbels were parasitized by *P. laevis* and *Contracaecum* larvae, two barbels by *P. laevis* and *B. rectangulum*, and three of them by *P. laevis* and *R. acus* larvae. Only two fish (2.17 %) were infected with three parasite species (*P. laevis*, *Contracaecum* sp. and *B. rectangulum*).
A maximum of 354 helminth specimens per fish host was detected. The average species richness (mean number of species in fish specimen) in infracommunities of barbel was 1.22 ± 0.46, the average abundance (mean number of helminth specimens in

Table 1. Basic characteristics of *Barbus barbus* examined from the River Danube within individual seasons.

Season	Number of fish	Mean body length and range (mm)	Mean body weight and range (g)
Spring	33	350.5 (290 – 450)	415.2 (220 – 788)
Summer	28	361.0 (290 – 445)	431.3 (285 – 635)
Autumn	31	371.0 (310 – 420)	491.3 (395 – 745)

fish) was 69.6, Brillouin diversity index HB = 0.075±0.079 (range 0.008 – 0.166). A comparison of the mean HB and the mean number of helminth species showed significant seasonal differences (Two-sample t-test, p = 0.00). The mean number of specimens of *P. laevis* was significantly higher than the number of specimens of all other species in each season (One-way ANOVA, p = 0.03).

Component community of helminth endoparasites of barbel

The vast majority (6,340 specimens) of the parasite component community of *B. barbus* composed two acanthocephalan species, followed by nematodes (49) and cestodes (19), with *P. laevis* as the most prevalent, core species (P = 98.9 %). *Contracaecum* sp. was proved as the second most frequent species (P = 14.1 %), while *B. rectangulum* (P = 4.4 %), *R. acus* (P = 3.3 %), and *A. anguillae* (P = 1.1 %) represented accidental species of the component community (Table 2).

The maxima of the mean intensity and mean abundance were detected in *P. laevis* in all seasons and thus, it represents the dominant species of the component community (Table 3). Significant differences were found between the number of *P. laevis* and the summary number of all other parasites in each season (Two-sample t-test, $p_{spring} = 0.002$, $p_{summer} = 0.03$, $p_{autumn} = 0.002$). The prevalence of *P. laevis* and other parasite species also differed significantly (One-way ANOVA, $p_{P. laevis/B. rectangulum} = 0.001$, $p_{P. laevis/A. anguillae} = 0.002$, $p_{P. laevis/Contracaecum sp.} = 0.006$, $p_{P. laevis/R. acus} = 0.002$). The same results were found for mean intensity of infection (One-way ANOVA, $p_{P. laevis/B. rectangulum} = 0.03$, $p_{P. laevis/A. anguillae} = 0.02$, $p_{P. laevis/Contracaecum sp.} = 0.01$, $p_{P. laevis/R. acus} = 0.02$).

Contracaecum larvae showed much lower infection values throughout seasons with the highest prevalence and intensity of infection in summer (Table 3). Remaining parasites occurred solely in summer and autumn (*B. rectangulum*), only in summer (*A. anguillae*) or only in autumn (*R. acus*), always with a low infection (Table 3).

The highest component species diversity was found in summer and autumn, when four parasite species regularly occurred in barbels (Table 3). In spring, only two parasite species were detected; all barbels were infected with *P. laevis* and the only fish harboured two *Contracaecum* larvae. Above two species also occurred in *B. barbus* in all investigated seasons. The only fish specimen free of *P. laevis* occurred in summer, but it was infected with *A. anguillae*. The differences between the seasonal patterns of the *P. laevis* prevalence were not significant, similarly as between the intensity of infection, which culminated in spring (MI = 78.9). In *Contracaecum* sp. nematodes, significant seasonal differences were observed in both, the prevalence (χ^2, p=0.04) and mean intensity of infection between spring and summer (Two-sample t-test, p = 0.00), spring and autumn (Two-sample t-test, p = 0.00) and summer and autumn (Two-sample t-test, p = 0.01). The mean intensity of infection of *B. rectangulum* was significantly higher in summer than in autumn (Two-sample t-test, p = 0.07).

Taking into account all parasites except for *P. laevis*, significant differences were found between their mean intensity of infection and mean number of parasite specimens in summer and autumn (Two-sample t-test, p = 0.02 and p = 0.008, respectively).

Shannon diversity index and Pielou's evenness index were relatively low, with the lowest values in the spring period and the highest in summer (Table 4). On the contrary, Berger-Parker dominance index was the highest in spring (0.999) and lowest in summer (0.972). The Percentage similarity index (Ics) showed the highest similarity of helminth component communities of barbel between the spring and autumn periods (Ics = 0.989), followed by that between summer and autumn (Ics = 0.981) and summer and spring (Ics = 0.973), but the differences were not significant.

Table 2. The prevalence (P) and intensity values of helminth parasites detected in 92 barbels *Barbus barbus* from the River Danube.

Helminth species	Number of fish infected by individual parasite	P (%)	Number of specimens	Mean abundance ± SD	Mean intensity ± SD (range)
Bathybothrium rectangulum	4	4.4	19	0.2 ± 1.1	4.8 ± 2.9 (1 – 9)
Acanthocephalus anguillae	1	1.1	5	0.05 ± 0.5	5.0 (5)
Pomphorhynchus laevis	91	98.9	6,335	68.8 ± 67.1	69.6 ± 67.1 (2 – 354)
Contracaecum sp. larvae	13	14.1	43	0.5 ± 1.6	3.3 ± 3.0 (1 – 12)
Raphidascaris acus larvae	3	3.3	6	0.06 ± 0.4	2.0 ± 0.8 (1 – 3)

Table 3. Species diversity of helminth parasites of *Barbus barbus* from the River Danube within three seasons (N - number of examined fish, P – prevalence (%), MI – mean intensity).

Helminth species	Spring (N = 33)		Summer (N = 28)		Autumn (N = 31)	
	P	MI (range)	P	MI (range)	P	MI (range)
Bathybothrium rectangulum	–	–	3.6	9.0 (9)	9.7	3.3 (1 – 5)
Acanthocephalus anguillae	–	–	3.6	5.0 (5)	–	–
Pomphorhynchus laevis	100.0	78.9 ± 67.8 (2 – 341)	96.4	60.1 ± 68.6 (2 – 354)	100.0	68.0 ± 63.5 (2 – 237)
Contracaecum sp. larvae	3.0	2.0 (2)	21.4	5.3 (3 – 12)	19.4	1.5 (1 – 2)
Raphidascaris acus larvae	–	–	–	–	9.7	2.0 (1 – 3)

Discussion

Even though the parasitofauna of various fish species from the Lower Danube River, including its Bulgarian part, had been relatively often investigated (see the Introduction), data on helminths of barbel *B. barbus* were infrequently recorded. Summary data of the Table 5 show that helminth communities vary more or less in individual years. For instance, the cestode *B. rectangulum* (Bothriocephalidea) had so far been reported only from *Barbus meridionalis* and *Barbus cyclolepis* in Bulgaria (Kakacheva-Avramova, 1983). We have found this parasite in the *B. barbus* that represents the new host record in the Lower Danube River and even in Bulgaria.

Compared to *B. rectangulum*, acanthocephalans *A. anguillae* and *P. laevis* (both Echinorhynchida) have much broader fish host spectrum in the Danube River. *Acanthocephalus anguillae* is a euryxenous parasite having a wide host range, which includes at least 40 fish species (Moravec, 2001). In the Lower Danube River, this species was commonly referred in *B. barbus* by Margaritov (1959, 1966), Kakacheva-Avramova (1977), Kakacheva *et al*. (1978), Nachev & Sures (2009), Djikanovic *et al*. (2010) and Atanasov (2012), but it also often occurs in the Upper and Middle Danube sections (Moravec 2001; Moravec *et al*., 1997; Djikanovic *et al*., 2012).

The other common thorny-headed worm *P. laevis* has often been reported from various fish hosts including barbel, through the whole Danube River flow (e.g. Molnár, 1970; Moravec et al. 1997; Moravec, 2001; Schludermann *et al*., 2003; Ondračková *et al*., 2005; Djikanovic *et al.* 2010, 2012). In Bulgarian part of the Danube River, *P. laevis* was found in as many as 20 fish species including *B. barbus* (Nedeva *et al*., 2003). More recently, Nachev & Sures (2009) and Nachev (2010) also showed *P. laevis* as very frequent species in this location.

Acanthocephalans of the genus *Pomphorhynchus* have been intensively studied during the past decades. In Europe, *Pomphorhynchus* species have shown a certain degree of variability in their morphological characteristics and behaviour, which caused difficulties with their correct identification. Nowadays, the comprehensive molecular study on the phylogeography of European populations of *P. laevis* and *P. tereticollis* (Rudolphi, 1809) has been done by Perrot-Minnot *et al*. (2018) and it appears that only *P. laevis* occurs through the main flow of the River Danube. The study additionally showed rare co-occurrence of these species in the same habitats, several small rivers and a lake from the Danube River Basin. In this respect, the recent findings of *P. tereticollis* in *Abramis brama* from the Bulgarian lake Srebarna, adjacent to the Danube River (Kirin *et al*., 2013; 2014) is of greatest interest and needs additional study (Špakulová, personal communication). The coexistence of *P. laevis* and *P. tereticollis* had also been documented both in fish and intermediate gammarid hosts in different localities of Europe (Perrot-Minnot, 2004; Westram *et al.*, 2011; Perrot-Minnot *et al*., 2018).

Nematode larvae were rarely referred in fishes of the Lower Danube; except of the recently found *Contracaeum* and *Raphidascaris* representatives, only *Eustrongylides* sp. and *Hysterothylacium* sp.

Table 4. Comparison of seasonal diversity of helminth communities of *Barbus barbus* from the River Danube.

Season	Spring	Summer	Autumn
Number of helminth species	2	4	4
Number of helminth specimens	2606	1669	2133
H' (Shannon, diversity index)	0.006	0.149	0.076
E (Pielou, evenness index)	0.009	0.107	0.055
d (Berger-Parker, dominance index)	0.999	0.972	0.988
Dominant species	*P. laevis*	*P. laevis*	*P. laevis*

Table 5. Overview of helminth species of *Barbus barbus* recorded in the lower section of the River Danube.

Authority Helminth species	Margaritov (1959)	Margaritov (1966)	Kakacheva-Avramova (1977)	Nachev & Sures (2009)	Atanasov (2012)	This study
Cestoda						
Bathybothrium rectangulum						●
Caryophyllaeus laticeps	●	●			●	
Caryophyllaeus fennica		●			●	
Monogenea						
Dactylogyruscarpathicus			●			
Dactylogyrusmalleus	●		●			
Dactylogyrussphyrna			●			
Diplozoon sp.			●			
Trematoda						
Metagonimus yokogawai larv.				●	●	
Diplostomum spathaceum larv.				●	●	
Diplostomum pseudospathaceum larv.					●	
Posthodiplostomum cuticola larv.				●		
Nematoda						
Rhabdochona hellichi			●	●	●	
Rhabdochona denudata					●	
Rhabdochona sulaki (=gnedini)		●				
Rhaphidascaris acus larv.						●
Pseudocapillaria tomentosa				●		
Eustrongylides sp. larv.				●		
Contracaecum sp. larv.						●
Hysterothylacium sp. larv.				●		
Acanthocephala						
Pomphorhynchus laevis	●	●	●	●	●	●
Acanthocephalus anguillae				●	●	●
Leptorhynchoides plagicephalus				●		

were found by Nachev & Sures (2009) and Nachev (2010). In Europe, larvae of the ascaridoid genus *Contracaecum* sp. (Rhabditida) often occurred in internal organs of a wide spectrum of mainly cyprinid and perciform fishes. Up to ten *Contracaecum* species are available at the moment, but the systematics of their larval stages is difficult and little elaborated (Moravec, 2013). Therefore, supplementary molecular analyses of our *Contracaecum* samples would be beneficial to confirm species determination. We suppose, however, that it could be *Contracaecum microcephalum* (Rudolphi, 1809), because it was repeatedly reported from several other fish species in the River Danube in Bulgaria (Shukerova, 2006; Shukerova *et al*., 2010; Chunchukova *et al*., 2016).

Raphidascaris acus larvae (Rhabditida) occur in many fish species of different families of wide Holarctic distribution, most often in cyprinids (Moravec, 2013). In Bulgarian part of the Danube River and the lake Srebarna, larvae of *R. acus* were to date found in three cyprinids, *Abramis brama*, *Alburnus alburnus, Squalius cephalus* and *Perca fluviatilis* (Shukerova 2010; Shukerova *et al*., 2010; Chunchukova *et al*., 2017). In Upper and Middle Danube sections, *R. acus* was found frequently in various fishes including barbels (e.g. Moravec *et al*., 1997; Moravec, 2001; Ondračková *et al.*, 2005). Regarding Bulgaria, the recent report of *R. acus* larvae in barbel represents the new geographic and host record.

Information about seasonal changes of the fish helminth community structure is of great value for any studies on fish parasites and ecological assessment of freshwater habitats. Considering the seasonal changes in the component community structure, it comprised four parasites in summer (*B. rectangulum*, *A. anguillae*,

P. laevis and *Contracaecum* sp.) and in autumn (*B. rectangulum*, *P. laevis, Contracaecum* sp. and *R. acus*) and only two helminths (*P. laevis* and *Contracaecum* sp.) in spring. Only *Contracaecum* larvae were found in all three seasons round, being accidental in spring (3.0 %), core in summer (21.4 %), and component species in autumn (19.4 %). Numerous, mainly cyprinid fish including barbel (genera *Abramis, Alburnoides, Alburnus, Barbus, Gobio, Rutilus*), represent intermediate or paratenic hosts of *Contracaecum* nematodes (Moravec *et al*., 1997).

In the present study, *P. laevis* was the most abundant (core) species during all studied seasons of two years. It dominated significantly all other helminths in the number of specimens, prevalence and mean intensity of infection. Seasonal differences between these indices were not significant, which partly coincide with a life cycle mode of *P. laevis*, which is not seasonally dependent, same as a composition of the food of barbel. Potential decline of infection rates of the parasite might be related to availability of gammarid intermediate host in preferred habitats. It should be remembered that this parasite, in high numbers, may significantly affect the health status of the host fish and can cause substantial loss and even destruction of its populations, including in aquaculture (Moravec et al., 1997; Gettová *et al*., 2016).

The stenoxenous *B. rectangulum* was classified as the parasite of barbel with an accidental incidence. Its mean intensity increased in summer, the maximum prevalence was quoted during autumn, but was completely absent in the study site in spring. In other Danube habitats, however, it occurred during the whole year including spring seasons (Scholz & Moravec, 1996; Moravec *et al.,* 1997). These authors described a seasonal cycle of this tapeworm and explained that worms with ripe eggs left the fish hosts from May to July. Reproduction cycle of *Bathybothrium* might be modified by specific local environmental conditions, which could the lack of the species recorded by us in spring. The host specificity of *B. rectangulum* discussed Kakacheva-Avramova (1983), who found this species also in *Gobio gobio* or *S. cephalus*, but the cestode had never reached sexual maturity in these fish hosts.

In the lower Danube, some helminth species seems to be rather rare. Only five specimens of the acanthocephalan *A. anguillae* were found in a single fish host screened in summer, and only six nematode larvae the *R. acus* were found in three barbels screened in autumn. The number of studies (for review see Moravec *et al*., 1997) showed clear seasonal dynamics in occurrence of this nematode with maximum in autumn, decrease in winter, second maximum in spring and minimum in summer. At the same time, significant variations were reported by the habitats, fish size and age and specific fish host diet.

Taking into account all parasites except of *P. laevis*, their mean intensity of infection and mean number of parasite specimens were significantly higher in summer comparing with autumn. The Brillouin diversity index (HB) was significantly higher in summer than in autumn, while the maximum values of Shannon diversity and the Pielou's evenness indices were documented higher in summer but contrary to HB, they were lowest in spring. These results indicate better biotic and environmental conditions for a majority of barbel parasites in summer seasons (Hudson *et al*., 2006). Evaluating the complex information including *P. laevis* data, most indices have changed significantly due to the unbalanced number of individual parasite species (significantly prevailing *P. laevis*) in the examined data set in all seasons. The Shannon diversity and the Pielou's evenness indices were low, while the Berger-Parker dominance and Sorensen similarity indices were relatively high (see Table 4). Berger-Parker dominance index was the highest in the spring period and lowest in summer, in accordance with the dominance of *P. laevis* in the data set.

Our study of endohelminth community of *B. barbus* from the Lower Danube (biotope Vetren) corresponds only partially with previous surveys by Margaritov (1959, 1966), Kakacheva-Avramova (1977), Nachev & Sures (2009), and Atanasov (2012) (Table 5). The spectrum of barbel parasites differed depending on the study site, number of examined fish, and various ecological factors like fish feeding habits and water quality. The number of helminth species, reported by above papers, ranged between three (Margaritov, 1959) and 10 taxa (Nachev & Sures, 2009). Caryophyllidean cestodes, acanthocephalans (mainly the dominating *P. laevis*) and nematodes (the obligate *Rhabdochona* spp.) were most common, while trematodes (e.g. *Diplostomum* sp. larvae) often absent and monogeneans were most probably rarely checked. Significant differences in the parasite diversity of barbel have been found between our study, other Danube River sites or areas geographically close to the Danube (Moravec *et al.,* 1997; Schludermann *et al*., 2003; Nachev, 2010). Relatively low parasite species diversity of barbel recorded during two years, and low values of most indices might indicate some negative environmental conditions in the studied area, which should be subsequently reviewed.

Conflict of Interest

Authors state no conflict of interest.

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Seasonal variation in helminth parasites of snakeheads *Channa punctatus* and *Channa striatus* (Perciformes: Channidae) in Uttar Pradesh, India

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Summary

Snakehead fishes are widely consumed throughout South East Asia, China and India because of their good taste of meat and high nutrient values such as presence of prostaglandins, thromboxane and Omega-6 fatty acid. Parasitic infection constitutes significant economic loss in fish production. The aim of this work was to study the seasonal variation of helminths in snakeheads. In the presented study, a three-year survey has been performed. A total of 1013 individuals of *Channa punctatus* and 247 individuals of *Channa striatus* were examined. A total of 3783 helminths were collected, with an average of 3.02 helminths/fish. 43.50 % individuals of *C. punctatus* and 59.10 % of *C. striatus* were found to be infected with acanthocephalans, trematodes, nematodes and cestodes per year. The prevalence and mean abundance of *Pallisentis* sp. was at its peak in summer. However the prevalence of trematodes, nematodes and cestodes was at peak during autumn. Mean abundance of nematodes was at peak in summer. Interestingly, the males were found more infected as compared to the females and the infection rate in males peaked in summer. In comparison to other weight groups, medium size hosts (21 – 40 g) were found more consistently infected. Thus the results indicate that there are seasonal variations in parasitic helminths infecting *C. punctatus* and *C. striatus* which also depend upon sex and weight. These variations may be attributed to various environmental and biological factors including parasite life cycle and immune level of host.
Keywords: nematode; fish; helminths; parasites; prevalence; snakeheads

Introduction

A large proportion of proteins obtained from animal sources come from fishes. Out of total worldwide protein obtained by animal sources, 25 % alone is contributed by fish and shellfish thus; fish is one of the most valuable sources of protein in food. About 80 % infection of warm water fishes is caused by parasites (Eissa, 2002). Helminths play a key role in internal parasitic infections of fishes which leads to low body weight gain and high mortality rate. Parasitic infection either alone or in conjunction with stress may reduce host weight and reproduction which leads to economic loss (Rohde, 1993). Parasites also upset the normal reproduction of the hosts (Faust, 1940).

The infection of parasites interferes with nutrition, metabolism and secretory function of the alimentary canal, damages nervous system (Markov, 1961) which may also lead to gastrointestinal abrasions and facilitate the invasion by opportunistic microorganisms. Unfavorable environmental conditions contribute to stress which also weakens the immunity and opens the pathway to pathogens (Eissa, 2002). The study of diversity and distribution of helminths

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started in the middle of the 19th century in India and numerous works has been done by Bhalerao, 1937; Gupta, 1984; Soota, 1981; Sood, 1989; Tondon *et al*., 2005; Bhure, 2008, Pandey & Agrawal, 2008; Deshmukh, 2015 in different parts of India.

The distribution of helminths is not only affected by seasons but also by host age, size, diet, abundance of fishes and an independent number of parasites within the fish. Change in climatic conditions is predicted to affect the prevalence of parasites in freshwater and marine ecosystems. A study of Chubb (1977 & 1979) showed the seasonal occurrence of helminths of freshwater fishes from different climatic zones.

Price and Clancy (1983) had also reported that larger bodied fish species harbor more parasitic diversity than small-bodied fish, but according to Sasal *et al*. (1997) there is no association between fish body size and parasite species richness. Available data on factors which potentially control the number of parasite species in freshwater and marine fishes (Sasal *et al*., 1997; Luque & Poulin, 2004) are very little in consistency. Moreover, available studies on the helminth parasitic fauna of snakeheads in relation to seasonal population dynamics, size and sex of the host are very little. The aim of this study was to investigate the seasonal variation of parasitic helminths of freshwater fishes from Uttar Pradesh, India with relation to host sizes and sex.

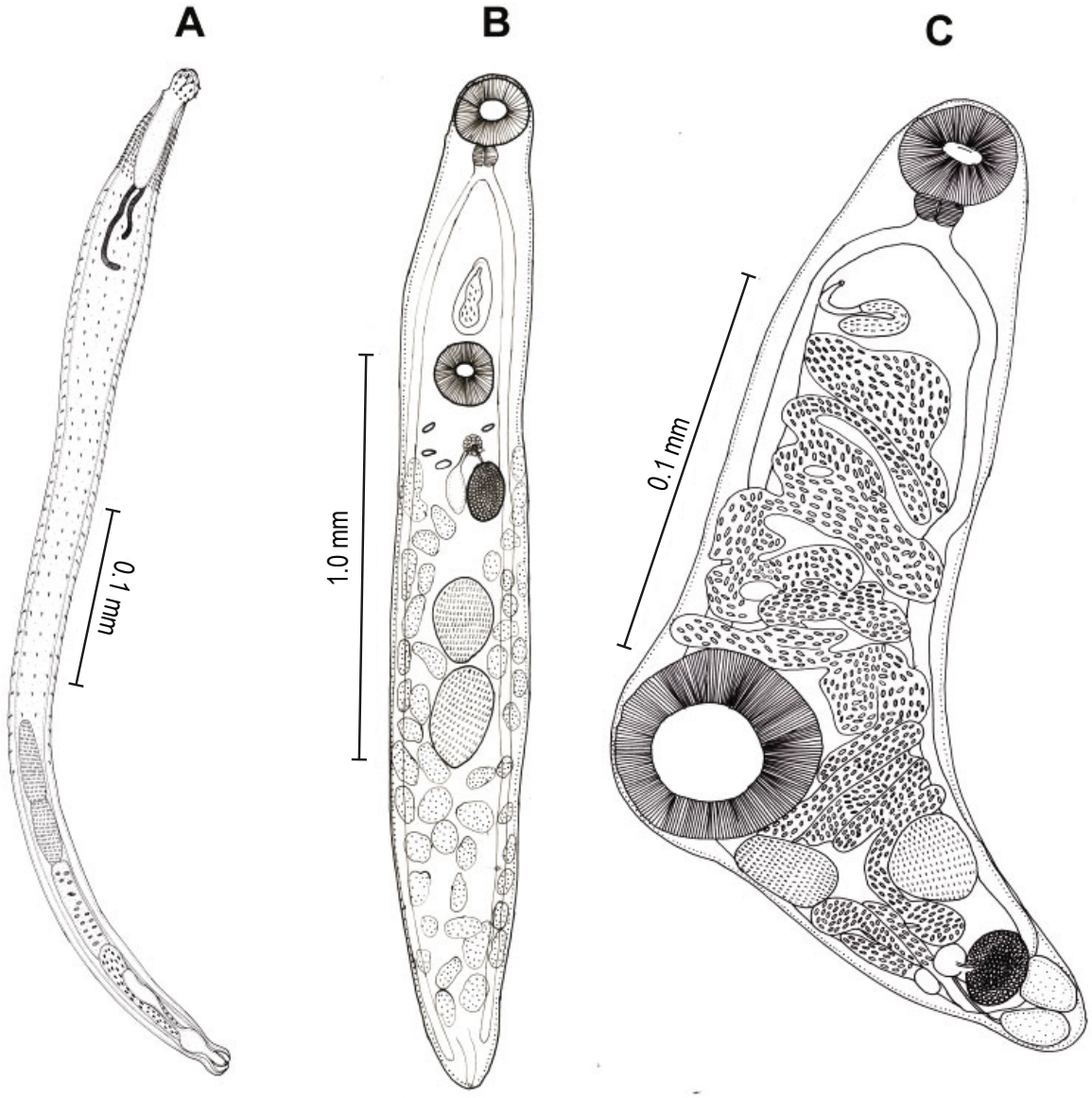

Fig. 1. Line drawings of Acanthocephalan and Trematodes: A) *Pallisentis* sp. B) *Allocradium* sp. C) *Genarchopis* sp.

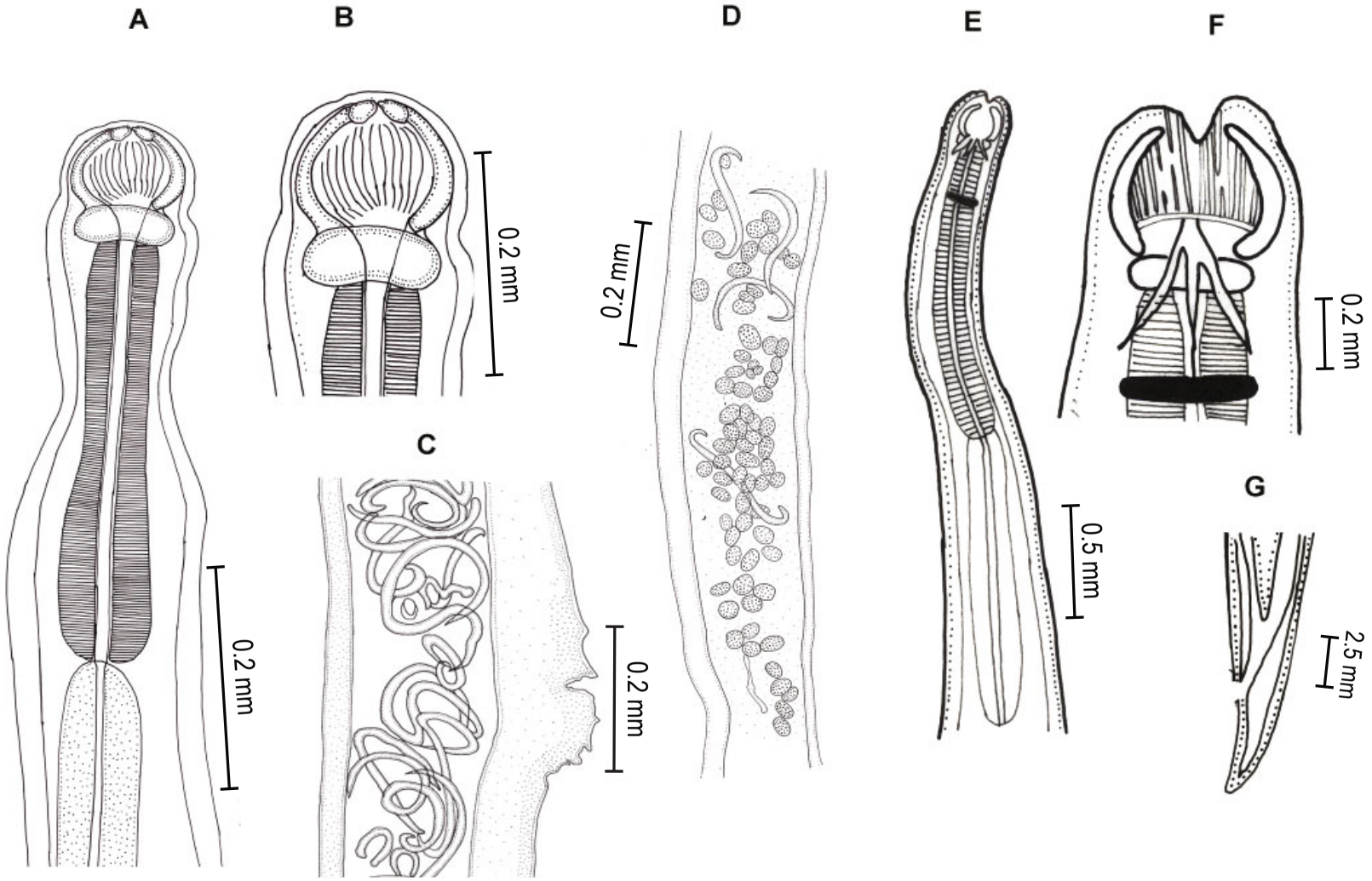

Fig. 2. Line drawings of Nematodes: A) anterior extremity of *Neocamallanus* sp. B) Buccal capsule of *Neocamallanus* sp. C) Vulvar region of female *Neocamallanus* sp. D) Gravid of female *Neocamallanus*. E) Anterior extremity of *Procamallanus* sp. F) Buccal capsule of *Procamallanus* sp. G) Vulvar region of female *Procamallanus* sp.

Materials and Methods

A three-year survey (September 2013 to August 2016) was performed. Fish samples were collected four times in a year during winter (December – February), autumn (September – November), summer (March – May) and rainy season (June – August). Fishes were collected from water bodies near Lucknow (26°84'67"N; 80°94'62"E). Fishes were weighed, measured and identified according to Vazzoler (1996).

Bodies were opened along the midventral line from the anal region to the mouth. The surface of the visceral organs, intestine and the body cavity were examined carefully. The alimentary canal was separated and kept in Petri dishes containing normal saline (0.9 %). The stomach and intestine were opened to dislodge parasites. A few drops of the methanol were added to the normal saline, containing the parasites adhered to the intestinal wall for immobilization and loosening of the grip on the intestinal wall.

Live specimens of parasites collected from the stomach and intestine of host and were kept in normal saline. Parasites were flattened with the help of a glass cover slip and fixed in AFA {Alcohol (50 %): formalin: acetic acid (100: 6: 2.5)}. Specimens were stained with acetoalum carmine, dehydrated in ascending grades of ethanol (30 %, 50 % 70 %, 90 % and absolute ethanol), cleared in xylene and mounted in DPX. Figures were drawn with

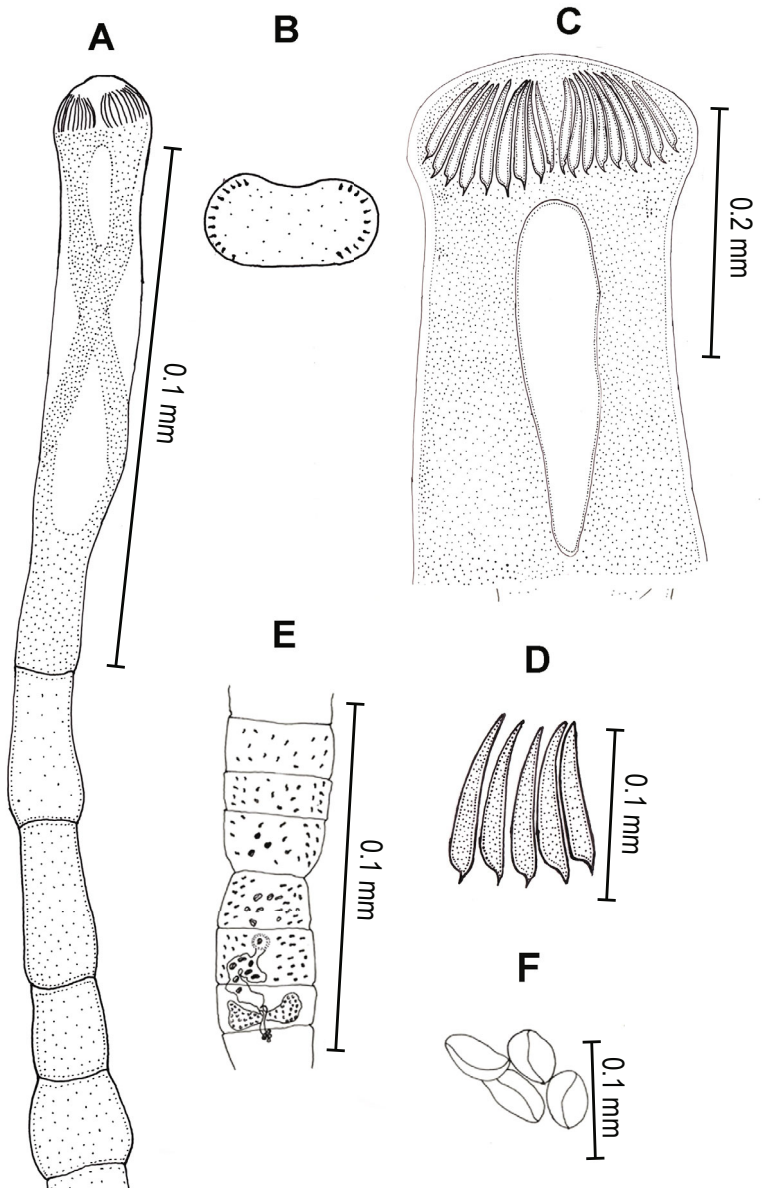

Fig. 3. Line drawings of *Senga* sp. A) Scolex and neck. B) Apical disc of scolex with hooks. C) Enlarge view of scolex. D) Enlarge view of scolex hooks. E) Mature proglottid. F) Eggs.

Camera Lucida, attached to Phase Contrast Microscope (Olympus CX-41). Parasites were identified up to genus level by using the keys of Yamaguti, 1958; Rego *et al*., 1999; Amin *et al*., 2000; Bhattacharya, 2007 and Gibbons, 2010. The slides of collected specimens were deposited in the "Helminthology lab, Department of Zoology, University of Lucknow, U.P., India. Collection numbers: LU/Z/2017/13, LU/Z/2017/14, LU/Z/2017/15, LU/Z/2017/16, LU/Z/2017/17 and LU/Z/2017/18 were assigned for *Pallisentis* sp., *Allocredium* sp., *Genarchopsis* sp., *Neocamallanus* sp., *Procamallanus* sp. and *Senga* sp. respectively.

Only the species that had prevalence equal or greater than 10 % in at least one of the collections were further included in the analysis. Prevalence, intensity range, mean intensity and mean abundance were calculated following Rohde *et al*. (1995). Possible differences between the prevalence in different seasons in relation to total samples were evaluated with Kruskal-Wallis H test .The Wilcoxon Sign Rank test was used for the analysis of infection in male and female. We set the null hypothesis, H_0: infection in male and female were equal. i.e. $p_{1=} p_2$. We also set alternate hypothesis, H_1: Infection in male and female were not the same. i.e. $p_1 \neq p_2$. From the given data we got interested in testing whether the infection in male and female *C. punctatus* and *C. striatus* are same or different.

The ecological terminology used was recommended by Bush *et al*. (1997). The level of statistical significance was $p < 0.05$.

Ethical Approval and/or Informed Consent

Animal care and handling were carried out in accordance with national guidelines.

Results

A total of 1013 individuals of *C. punctatus* and 247 individuals of *C. striatus* were examined. Overall 3783 helminths were found with an average of 3.02 helminths/fish collected. On an average 43.50 % *C. punctatus* and 59.10 % *C. striatus* were found to be infected with Acanthocephalans (*Pallisentis* sp., Fig.1A), trematodes (*Allocredium* sp. Fig.1B and *Genarchopsis* sp. Fig.1C), nematode (*Neocamallanus* sp. Fig.2A–2D and *Procamallanus* sp. Fig.2E–2G) and cestodes (*Senga* sp. Fig.3A–3F) per year.

The prevalence, intensity range, mean intensity and mean abundance of various helminths in *C. punctatus* and *C. striatus* were observed (Table 1). The digestive tract was found to be harbouring one acanthocephalan, two trematodes, two nematodes and one cestode genus. Acanthocephalan *Pallisentis* spp. was the most prevalent and abundant parasite (46.50 % infected specimen in *C. punctatus* and 59.11 % in *C. striatus*). We could find only one cestode *Senga* sp. in both *C. punctatus* and *C. striatus*

The highest mean intensity and abundances were found in *Pallis-*

Table 1. Prevalence, intensity range, mean intensity and mean abundance of helminths of snake headed fishes in Uttar Pradesh, India.

	Parasites	Prevalence (%)	Intensity range	Mean Intensity ± SD	Mean Abundance ± SD	Site of infection
C. punctatus	**Acanthocephala**					
	Pallisentis sp.	46.50	12.33 – 0.64	3.79 ± 3.31	1.70 ± 1.52	Intestine
	Trematode					
	Allocredium sp.	21.74	2.78 – 0.00	0.97 ± 0.79	0.23 ± 0.25	Intestine
	Genarchopsis sp.	19.66	3.68 – 0.17	1.31 ± 0.98	0.30 ± 0.28	Stomach
	Nematode					
	Procamallanus sp.	14.53	3.83 – 0.00	1.38 ± 0.95	0.25 ± 0.25	Intestine
	Cestode					
	Senga sp.	13.04	2.25 – 0.00	1.21 ± 0.68	0.20 ± 0.21	Stomach and Intestine
C. striatus	**Acanthocephala**					
	Pallisentis sp.	59.11	20.21 – 0.13	5.11 ± 6.92	3.42 ± 5.98	Intestine
	Trematode					
	Allocredium sp.	26.32	7.78 – 0.00	2.00 ± 2.21	0.56 ± 0.78	Intestine
	Genarchopsis sp.	18.62	3.22 – 0.00	1.15 ± 1.01	0.28 ± 0.43	Stomach
	Nematode					
	Neocamallanus sp.	17.81	7.60 – 0.00	1.70 ± 2.11	0.36 ± 0.51	Intestine
	Cestode					
	Senga sp.	14.57	7.50 – 0.33	2.15 ± 2.37	0.27 ± 0.31	Stomach and Intestine

Table 2. Seasonal differences in the prevalence (%) (Mean ± SD) of helminths in snakehead fishes U. P. India.

Host	Parasites	Acanthocephala	Trematode		Nematode	Cestode
	Seasons	*Pallisentis* sp.	*Allocredium* sp.	*Genarchopsis* sp.	*Procamallanus* sp.	*Senga* sp.
C. punctatus	**Autumn**	68.85 ± 3.83	18.19 ± 4.12	22.35 ± 5.96	22.09 ± 18.62	29.93 ± 21.97
	Winter	34.79 ± 9.68	25.15 ± 13.96	15.67 ± 4.15	13.94 ± 7.98	11.66 ± 6.58
	Summer	65.21 ± 5.90	27.71 ± 5.93	22.19 ± 6.17	18.38 ± 2.94	12.03 ± 1.10
	Rainy	36.47 ± 11.64	26.26 ± 10.00	24.87 ± 9.68	20.30 ± 7.41	13.28 ± 3.64
	Chi-square	8.641	2.487	1.974	1.564	1.051
	p-value	0.034	0.478	0.578	0.668	0.789
		Pallisentis sp.	*Allocredium* sp.	*Genarchopsis* sp.	*Neocamallanus* sp.	*Senga* sp.
C. striatus	**Autumn**	66.72 ± 5.23	37.02 ± 15.00	34.48 ± 12.36	25.48 ± 9.97	18.87 ± 6.62
	Winter	22.22 ± 11.11	11.11 ± 11.11	25.93 ± 16.97	7.41 ± 6.42	18.52 ± 6.42
	Summer	79.97 ± 9.50	28.79 ± 4.67	2.66 ± 2.43	19.46 ± 6.49	10.14 ± 2.51
	Rainy	40.15 ± 6.44	16.53 ± 6.93	14.69 ± 9.28	11.60 ± 2.34	13.35 ± 5.78
	Chi-square	9.974	7.667	7.821	7.539	5.539
	p-value	0.019	0.053	0.050	0.057	0.136

entis sp. while the trematode *Allocredium* sp. and cestode *Senga* sp. were least abundant in *C. punctatus* and *C. striatus* respectively. There were statistically significant differences of prevalence between different seasons of *Pallisentis* spp. in *C. punctatus* (p = 0.034) and *C. striatus* (p = 0.019). But there were no significant differences in other helminths in either *C. punctatus* or *C. striatus* (Table 2). The prevalence of *Pallisentis* spp. was maximum in autumn and summer in *C. punctatus* (68.85 ± 3.83 and 65.21 ± 5.90 respectively) while in *C. striatus*, prevalence of *Pallisentis* spp. was maximum during summer (79.97 ± 9.50).

Mean abundance of most of the helminths species was higher during summer and never reached maximum observed during winter season (Table 3). Acanthocephalans and nematodes in both the host attained peak mean abundance during summer while trematode *Genarchopis* sp. in *C. punctatus* and *Allocradium* sp. in *C. striatus* showed maximum during rainy season. Only cestode *Senga* sp. in *C. punctatus* and trematode *Genarchopis* sp. in *C. striatus* showed maximum mean abundance during autumn.

Infection rates of parasitic helminths were observed for different sexes in changing seasonal conditions to study the effect of sex on infection. There was a statistically significant difference between the infection rates of male and female *C. punctatus* and C. *striatus* (Table 4). During all the seasons except autumn in *C. punctatus* and throughout all the seasons in *C. striatus* there were significantly higher infected males as compared to females.

Comparing different weight groups in hosts, we found that there was a tendency in medium weight group (21 – 40 g) to become more infected. In C. *punctatus* weight group 60 – 200 was infected most while as regards to the *C. striatus* in 21 – 40 weight group it was autumn season (Table 5). In winter season the infection rates were higher in the weight group of 21 – 40 for both C. *punctatus* and *C. striatus*. The infection rates in summer season were highest in weight group 41 – 60 in C. *punctatus* and in weight group 00 – 20 in *C. striatus*. In the rainy season, the highest prevalence of the infection was in the weight group 21 – 40 and 41 – 60 in C. *punctatus* and *C. striatus* respectively.

Discussion

The present study on two snakehead fishes was aimed to survey the occurrence and distribution of endoparasitic helminths and to explore the seasonal prevalence of these parasites. Six genera of helminths were found from trematode, cestode, nematode and acanthocephalans. Most of the parasites were found in the intestine region and the acanthocephalan *Pallisantis* spp. were most prevalent ones.

Seasonal variations of parasites in the host are already well studied. Distinct seasonal variation was reported by Boping and Wang (2007) and they found that the prevalence of *Pallisentis caelatus* (Neosentis) was at the highest in spring and decreased with fall in temperature. Earlier, Kanth and Srivastava (1987) had also reported that infestation rate of *Pallisentis ophiocephali* gradually increases and achieves two peaks in May and August. In our study, the infection rate differed seasonally and the maximum parasites were generally found during autumn and summer seasons. Among the nematode and cestode, peak prevalence was in autumn in both hosts, following the end of the peak breeding period of the host fishes. The Prevalence of trematode was not similarly regular

Table 3. Seasonal differences in mean abundance of helminths of snakeheaded fishes in U. P. India.

Host	Parasites	Total	Autumn	Winter	Summer	Rainy
C. punctatus	**Acanthocephala**					
	Pallisentis sp.	0.96	0.72	0.66	0.89	0.85
	Trematode					
	Allocredium sp.	0.54	0.40	0.33	0.72	0.58
	Genarchopsis sp.	0.50	0.46	0.40	0.55	0.60
	Nematode					
	Procamallanus sp.	0.54	0.53	0.42	0.58	0.54
	Cestode					
	Senga sp.	0.46	0.62	0.44	0.45	0.33
C. striatus	**Acanthocephala**					
	Pallisentis sp.	0.82	0.75	0.66	0.85	0.85
	Trematode					
	Allocredium sp.	0.46	0.31	0.40	0.66	0.80
	Genarchopsis sp.	0.65	0.88	0.33	0.60	0.66
	Nematode					
	Neocamallanus sp.	0.30	0.23	0.22	0.50	0.33
	Cestode					
	Senga sp.	0.33	0.35	0.40	0.42	0.18

and differed in both hosts. For *C. striatus* the peak was in autumn but *C. punctatus* attained its peak in both summer and rainy seasons. Many authors have also reported that high prevalence of cestode (Bhure *et al*., 2014) in summer whereas low in the monsoon season. Similarly Vincent and Font (2003) reported that the prevalence, mean abundance and mean intensity of nematodes were higher in summer than in winter. According to Genc *et al*., (2005) the parasitic infection showed seasonal variations with the highest prevalence in the summer season.

Two main categories of factors may be held responsible for the seasonal variations in host infectivity, those linked to the host and other linked to the parasites. Ibiwoye *et al*., (2004) observed that susceptibility to infections in fishes are generally due to weakened body after hibernation. According to Bhuiyan *et al*., (2007) decrease in water volume during dry seasons results in imbalanced nutritional conditions also make fishes vulnerable to the infections. The authors also concluded that decreased water temperature also made the hosts susceptible to infections by weakening immune systems. So many other parasite associated factors are also held responsible for the development of parasites such as high temperature and

Table 4. Seasonal differences of helminths of *C. punctatus* and *C. striatus* during different seasons.

Host	Seasons	Male	Female	Z	p*
C. punctatus	Autumn	6.22 ± 6.01	2.33 ± 1.73	1.90	0.057
	Winter	6.11 ± 6.60	0.44 ± 0.72	2.03	0.042
	Summer	20.33 ± 18.01	4.55 ± 2.35	2.36	0.018
	Rainy	5.22 ± 7.67	1.88 ± 3.14	2.13	0.033
C. striatus	Autumn	4.33 ± 2.23	1.66 ± 1.22	2.55	0.011
	Winter	2.11 ± 1.26	1.11 ± 1.05	2.04	0.041
	Summer	6.66 ± 7.36	0.88 ± 1.36	2.37	0.018
	Rainy	2.66 ± 2.00	0.88 ± 1.36	2.45	0.014

Z value of Kruskal-Wallis H test, *significance level $p \leq 0.05$

Table 5. Helminths infecting *Channa punctatus* and *Channa striatus* of different weight groups as 00 – 20 g, 21 – 40 g, 41 – 60 g and 61 – 200/300 g.

		Weight (g)			
Host	**Seasons**	**00 – 20 (p_1)**	**21 – 40 (p_2)**	**41 – 60 (p_3)**	**60 – 200/300 (p_4)***
C. punctatus	Autumn	26.3	48.75	7.69	70.0
	Winter	18.30	51.28	31.25	13.33
	Summer	50.0	52.90	59.23	51.28
	Rainy	36.20	78.76	39.47	0.00
C. striatus	Autumn	64.15	76.08	50.0	0
	Winter	46.66	75.55	71.42	50.0
	Summer	90.0	73.33	34.48	11.53
	Rainy	0	26.66	32.14	11.76

**Channa punctatus* maximum weight was 200 g and *Channa striatus* 300 g.

low rainfall (Jadhav & Bhure, 2006). Kennedy (1970 & 1977) had explained that feeding habits of the host, availability of infective host and parasite maturation are also responsible for influencing the parasitic infections. Recently, Sheema *et al*., (2015) and Ritika *et al*., (2012) have suggested that abundance of helminths increase with the rising temperature in summer and slow down during winter. The development of intermediate hosts of helminths during summer season also leads to better availability of infective stages resulting in higher helminths prevalence in summer (Khurshid & Ahmad, 2012).

During our study we also found significant differences in infestation rates on the basis of sex and weight groups. In overall four different seasons, the infestation rate in male C. *punctatus* was higher in the summer. The association between reproduction and increase in prevalence and abundance of parasites has been attributed the fact to the physiological stress of the host during the breeding period, as a higher investment in reproduction may decrease the energy allocated to the immune system and thereby facilitate parasitic infestation (White *et al*., 1996; Lizama *et al*., 2006). There are contrasting reports in this regard as some authors have observed more infection in male hosts as compared to females (Zelmer & Arai, 1998) and this view is supported by our study. Other observers have reported a greater susceptibility of females (Ibiwoye *et al*., 2004; Singhal & Gupta, 2009). These differences may be because of various factors including host species, infective species and geographical conditions.

Variations in infection rates in different weight group were less profound in summer where in the rest seasons, there were more variations. Overall, the mid-weighed snakeheads were more prone to infection as compared to lower and higher weight groups. Our results are in accordance with some earlier observations. Nahar (1988) have also reported similar finding that the hosts with intermediate size were more infected by the parasites than the smaller and larger individuals. Similar reports by Polyanski (1961) had supported the view that intermediate length and weight group host had a higher prevalence and intensity than those of smaller and larger length and weight group. Major factors such as food, lifespan, variety of habitats, population density and size attained by host were suggested to affect the parasites prevalence and intensity in fish.

Our study explores the diversity and seasonal variations of helminthic parasites in snakeheads. Our study explores the diversity and seasonal variations of helminthic parasites in snakeheads. These types of studies will lead to the better understandings of host-parasite interactions what will be beneficial for the improvement of infectious diseases management and also contribute to the increase in fish production.

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Conflict of Interest

Authors state no conflict of interest.

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Case Report

First report of *Athesmia foxi* Goldberger and Crane, 1911 (Digenea, Dicrocoeliidae) from *Chrysocyon brachyurus* (Illiger, 1815) (Carnivora, Canidae) and pathological findings

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Summary

Chrysocyon brachyurus, the largest South American canid, is a native species of the Brazilian cerrado. The present study is aimed to report the occurrence of the trematode, *Athesmia foxi,* in the liver of a new host, *C. brachyurus*, and to describe its morphology and pathology. One *C. brachyurus* individual was necropsied and examined for the presence of parasites. Worms were collected from the bile ducts and based on morphological and morphometrical characteristics, such as a relatively large, slender, aspinose, elongated shape with vitellarium present on the upper left side of the body were identified as *A. foxi*. On the host, hepatic lesions limited to the bile ducts and periportal regions, were characterized as chronic-active cholangitis, biliary hyperplasia, and fibrosis . This is the first report of *A. foxi* parasitizing *C. brachyurus*, demonstrating that this parasite has no host specificity and can be widely distributed. *A. foxi* lesions noted in *C. brachyurus* are similar to those noted in various other mammalian hosts.

Keywords: Digenea; Dicrocoeliidae; *Athesmia foxi*; Canidae; *Chrysosyon;* histopathology; Brazil

Introduction

The maned wolf, *Chrysocyon brachyurus* (Illiger, 1815), is the largest canid from South America and is the only species of its genus. It is a native species of the Brazilian cerrado, where it feeds on small animals and wild fruits (Nowak, 1991). The maned wolf is listed as near threatened in the IUCN's Red List of 2017. The decline in the population of this species is due to poaching and habitat destruction (Cavinato, 1999).

The taxonomy of the genus *Athesmia* needs to be better clarified. There are 12 species in the genus, being ten reported in birds and two in mammals. These species have morphological similarities that make it difficult to differentiate between them. There are conflicts over the validity of many of these species. Travassos (1944) considered the separation of the species according to geographic distribution in a broad sense, combined with their individual hosts. However, Freitas (1962) and Byrd *et al*. (1967) consider the genus as monotypic, with *A. heterolechithodes* being the unique species of the genus, considering all other proposed species within this one.

The present study aimed to report the occurrence of *A. foxi* in the liver of a new host, *C. brachyurus*, and to describe its morphology and pathology.

Material and Methods

One maned wolf, *C. brachyurus*, from Rio de Janeiro, Brazil, was necropsied and examined for the presence of endohelminths. Trematodes found in the bile ducts were collected and relaxed in saline solution. The worms were fixed in hot AFA (93 parts of 70 %

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ethanol; 5 parts formaldehyde; 2 parts glacial acetic acid) for 48 h, and then stored in 70 % ethanol. Digeneans were stained with Semichon's carmine, dehydrated using a graded ethanol series, cleared in glove oil, and mounted in Dammar gum. Measurements were performed on only three relaxed specimens because the other seven specimens were wrinkled, what might interfere with proper evaluation. Measurements of eggs (n=20) were taken from normal-appearing eggs in flat profile from the distal end of the uterus. Measurements are given in micrometers (unless otherwise stated), with mean ± standard deviation followed by a range in parentheses. Measurements were performed using an Axioplan Zeiss light microscope (Carl Zeiss, Germany) equipped with a Canon Power-Shot A640 digital camera (Canon, China), and Zeiss AxionVision Sample Images Software (Carl Zeiss, Germany) was used for the image analysis. Drawings were performed with the aid of an Axioplan Zeiss light microscope (Carl Zeiss, Germany) equipped with a camera lucida and were digitized using Adobe Photoshop Elements 8.0 software with the aid of an Intuos4 Wacon® pen tablet (Wacon Co. Ltd, Japan).

Specimens were deposited in the Helminthological Collection of the Oswaldo Cruz Institute (CHIOC), Fundação Oswaldo Cruz, Rio de Janeiro, Brazil (CHIOC nº 38867 a-c).

Ethical Approval and/or Informed Consent

The research was complied with all the relevant national regulations and institutional policies for the care and use of animals, according to federal resolution of veterinary medicine nº 829, from April, 25th, 2006. The maned-wolf was received by the Universidade Estadual do Norte Fluminese's Veterinary Hospital after being hit by a motor vehicle on a highway, where he died.

Results

Flukes collected from the bile ducts of a *C. brachyurus* were identified as *A. foxi* (Fig. 1) based on morphological and morphometrical characteristics (Table 1). Worms were characterized with a relatively large, slender, aspinose, elongated body (Fig. 1A). The following measurements were obtained: body length 10.1 ± 1.3 (8.8 – 11.3) mm; maximum width 851 ± 58 (785 - 890) measured at midbody level; oral sucker 452 ± 135 (322 – 592) long by 427 ± 149 (310 – 594) wide (ratio 1:1); distance from oral sucker to anterior extremity 1,637 ± 411 (1,257 – 2,074); ratio of pharynx width to oral sucker width approximately 1:3; ventral sucker 295 ± 32 (264 – 328) long by 316 ± 69 (265 - 395) wide (ratio 1:1); prepharynx absent; pharynx 124 ± 3 (122 – 127) long by 101 ± 13 (91 – 116) wide; esophagus 408 ± 88 (334 - 505) long. Cecal bifurcation was near to midlevel of forebody and anterior to genital pore (Fig. 1a). Ceca were similar in length, ending after the posterior end of vitellaria. Testes were lobed, tandem, and situated at midlevel of the upper half of the body. Anterior testes measured 494 ± 104 (424 – 615) long by 388 ± 58 (322 – 433) wide. Posterior testes were longer than they were wide, 662 ± 101 (584 – 775) long by 393 ± 45 (357 – 443) wide. Medial cirrus sacs measured 557 ± 120 (443 – 683) long by 101 ± 10 (89 – 107) wide, measured at the base, situated between the cecal bifurcation and ventral sucker, short cirrus and short ejaculatory duct and were surrounded by prostate cells (Fig. 1b). Intertesticular distance was 235 ± 90 (167 – 337). Genital pore near cecal bifurcation (Fig. 1b), was noted on the midline of the body. Distance of the posterior testes to the ovary was 547 ± 51 (491 – 591), representing 5.4 % of total body length. The ovary was lobed (Fig. 1c), measuring 430 ± 115 (308 – 537) long by 358 ± 44 (334 – 417) wide, was post-testicular and was located

Table 1. Comparisons of measurements in µm of Athesmia foxi Goldberger and Crane, 1911.

Characteristic	*Athesmia foxi*		
	Present study	**Goldberg; Crane, 1911**	**Stunkard, 1923**
Total body length	10,144 (8,800 – 11,300)	6,600 – 8,000	7,000 – 10,000
Body width	851 (785 – 890)	855	500 – 750
Oral sucker length	452 (322 – 592)	262	240 – 290
Oral sucker width	427 (310 – 594)	–	230 – 260
Pharynx length	124 (122 – 127)	82	80 – 100
Esophagus length	408 (334 – 505)	180 – 340	–
Ventral sucker length	295 (264 – 328)	340	200 – 230
Ventral sucker width	316 (265 – 395)	210 – 220	180 – 210
Cirrus sac length	557 (443 – 683)	1/6 of body	875 – 1,250
Cirrus sac width	101 (89 – 107)	–	200 – 270
Anterior testis length	494 (424 – 615)	–	40 – 50
Anterior testis width	388 (322 – 433)	510 – 540	430 – 600
Posterior testis length	662 (584 – 775)	420 – 520	360 – 500
Posterior testis width	393 (347 – 443)	480 – 540	430 – 600
Ovary length	430 (308 – 537)	460 – 520	360 – 500
Ovary width	368 (334 – 417)	–	270 – 370
Eggs length	37 (36 – 39)	34	27
Eggs width	20 (18 – 21)	20	19
Host	*Chrysosyon brabhyurus*	*Cebus capucinus*	*Cebus apella*

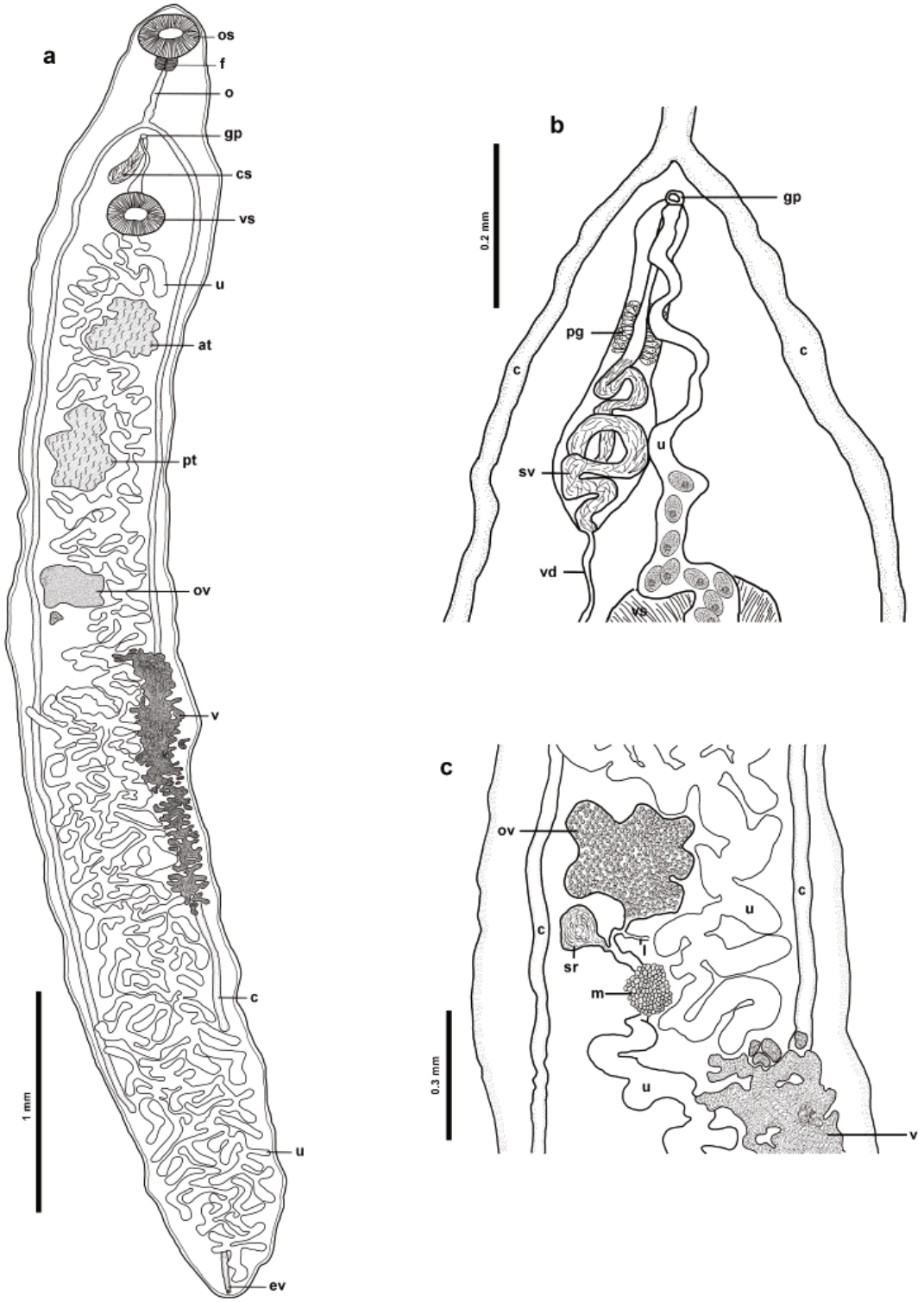

Fig. 1. *Athesmia foxi* from the maned Wolf, *Chrysocyon brachyurus* Illiger, 1815 (Carnivora, Canidae), from Rio de Janeiro, Brazil. (a) Ventral view of fully mature adult; (b) Cirrus sac and seminal vesicle in ventral view; (c) Female ge nital complex, ventral view. os - oral sucker; f – pharynx; o – oesophagus; gp – genital pore; cs – cirrus sac; vs – ventral sucker; u – uterus; at – anterior testis; pt – posterior testis; ov – ovarium; v – vitellaria; c – cecum; ev – excretory vesicle; pg – prostate gland; sv – seminal vesicle; vd – vas deferens; sr – seminal receptacle; l – Lauren's gland; m – Mehlin's gland.

approximately at the midlevel of the body. The seminal receptacle was rounded and located posterior to the ovary (Fig. 1c). Laurer's canal was present, arising from the oviduct across from the seminal receptacle, with the opening not observed. There was a single dendritic vitelline field (Fig. 1a), and vitellarium were present on the left side of the body, located in the upper half of the posterior half of the body, measuring 2,129 ± 253 (1,868 – 2,374) long and representing 21 % of total body length. Mehlin gland was present, located posterior to the ovary, at the anterior end of the vitelline field. The distance from the posterior end of the vitelline field to the posterior end of the body was 2,811 ± 350 (2,578 – 3,213). The uterus was large, highly coiled, and postacetabular, filling most of the hindbody (Fig. 1a). Numerous operculated eggs were noted, measuring approximately 37 ± 1 (36 – 39) long by 20 ± 0.8 (18 – 21) wide. Excretory vesicle was long, extending to the midbody region, and I-shaped; with a terminal excretory pore.

Histopathology

Histopathologic examination revealed hepatic lesions limited to the bile ducts and periportal regions. The bile ducts were widely distended and thickened due to proliferation of fibroblasts, fibrocytes and intense collagen deposition. Some ducts were occluded

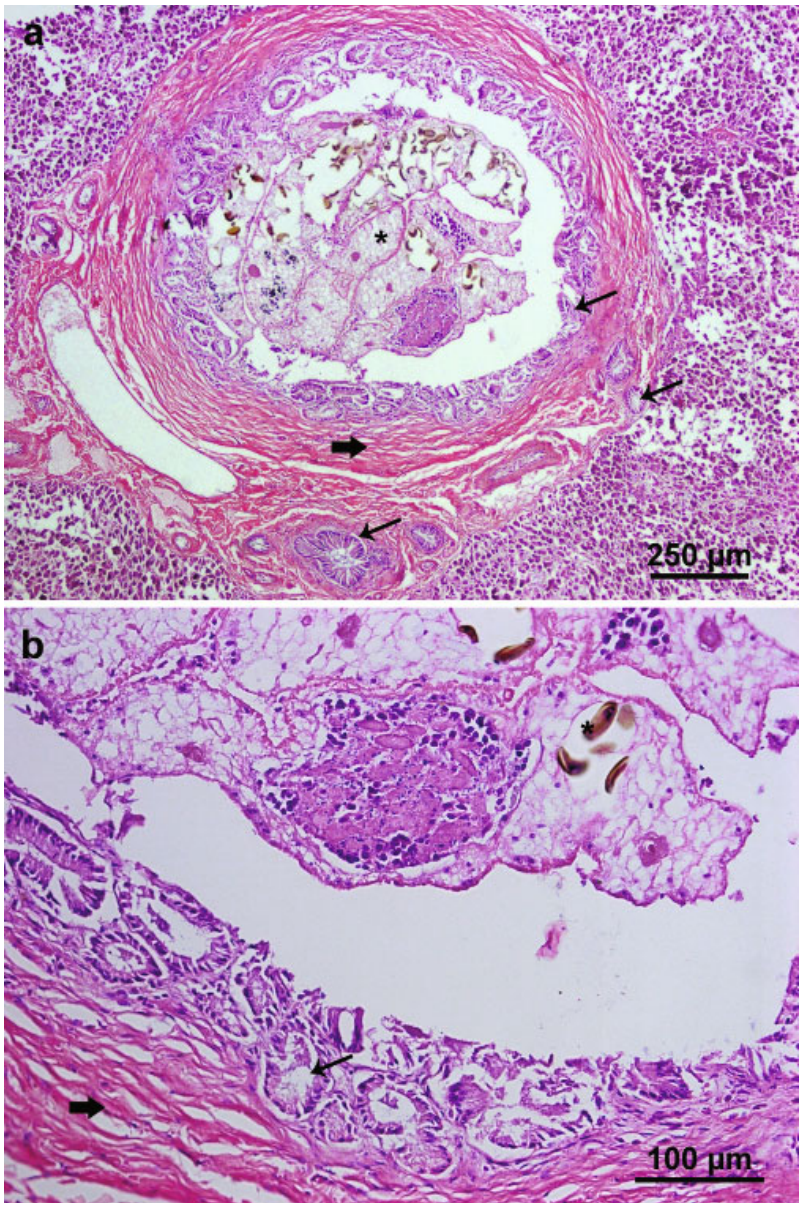

Fig. 2. Histological finding from *Chrysocyon brachyurus* Illiger, 1815 (Carnivora, Canidae) liver. (a) Bile duct containing several trematodes (*), showing formations of several ducts around the main duct (thin arrow) and fibrosis (large arrow); (b) Details of a bile duct showing formations of several ducts (thin arrow), fibrosis (large arrow) and parasites filled with eggs.

by up to six parasites filled with eggs, while other ducts contained free eggs and cell debris from the ductal epithelium. The epithelium was proliferative and reactive. There were formations of several ducts surrounding the main duct. Chronic-active inflammation was characterized by the presence of marked eosinophils and rare lymphocytes in the biliary epithelium and ductal wall. In this way, we can infer that *A. foxi* causes chronic-active cholangitis, biliary hyperplasia and fibrosis in the liver of *C. brachyurus*.

Discussion

Athesmia foxi was the first species of its genus described to infect mammals, and was noted for the first time in the liver of the South American primate *Cebus capucinus*. Subsequently, another species, *A. parkeri,* was described from a frugivorous bat, *Artibeus jamaicensis*. All other 10 species of the genus are reported to infect birds. *Athesmia foxi* has been seen in several mammal species South America, mainly in primates such as *C. capucinus* (Goldberger & Crane, 1911; Sawyer & Cheever, 1962; Faust, 1967), *Cebus apella* (Stunkard, 1923; Faust, 1967), *Callicebus cupreus* (Strong *et al*., 1926), *Chiropotes albinas* (Freitas, 1962), *Oedipomidas oedipus* (Caballero *et al*., 1952; Faust, 1967), *Saimiri sciurea* and *Cebus albifrons* (Faust, 1967). In addition, other non-primate mammals such as *Procyon cancrivorus* (Freitas, 1962) and *Rattus argenticenter* (Lee, 1965) have been reported as infected by *A. foxi*. Travassos (1942) established the genus *Pseudoathesmia* for specimens collected from the bile ducts of the canid *Cerdocyon thous*. In this genus, the vitellaria has a limited extension, not exceeding the intestinal cecum, which ends in the post-ovarian region, differing from the genus *Athesmia* that has a long cecum that extends beyond the post-ovarian region, and vitellaria extending along and surpassing the cecum (Travassos, 1944), concordant with the specimens collected from *C. brachyurus* in the present study.

The specimens collected from *C. brachyurus* and described by Faust (1967) appear to be different from *A. foxi* noted in this study, as well as from the reports of other authors. It is due the distribution of the uterus characterized in his study, which does not reach the posterior region of the body and ends approximately at the level of the end of the cecum. In the original description and in other studies regarding *A. foxi* (Stunkard, 1923; Travassos, 1944), the uterus occupies the region between the ventral sucker and the posterior extremity of the body (Fig. 1a).

Dronen (2014) divided the species of the genus *Athesmia* into two groups based on the posterior extension of the cecum in relation to the field occupied by the vitellaria: the attilae body type, in which the cecum is unequal, but both extend to at least near the level of the posterior end of the vitelline field or surpass it posteriorly (*A. attillae*, *A. butensis*, *A. ralli*, *A. reelfooti*, *A. wehri*). This is apart of the heterolechithodes body type, in which the cecum on the side of the vitelline field ends at the posterior margin of the vitelline field, usually near its anterior margin, whereas the opposing side's cecum extends to at least the level of the posterior margin of the vitelline field, generally surpassing it posteriorly (*A. foxi*, *A. parkeri*, *A. heterolechithodes*, *A. jolliei*, *A. kassimovi*). According to Dronen (2014), the two species of the genus *Athesmia* that infect mammals (*A. foxi* and *A. parkeri*) present the heterolechithodes body type, *i.e.,* with different cecum lengths, one longer than other. However, in the original description and in other studies performed on *A. foxi* (Stunkard, 1923; Travassos, 1944), both caeca exceed the vitelline field, characteristic of the attilae body type described by Dronen (2014).

Stunkard (1923) collected *A. foxi* from *Cebus apella* and described differences in the extent of the cecum in some specimens. Usually, one side of the cecum extends more posteriorly than the other. However, Stunkard (1923) stated that either cecum may be longer, and both may exceed the vitelline field. In a specimen collected by Stunkard (1923), the cecum on the vitelline side ends 0.74 mm before the caudal margin of the vitellaria, whereas the cecum on the opposite side extends 0.12 mm caudally to the vitellaria. In another specimen, the cecum on the vitelline side extends 0.54 mm posterior to the vitellaria field, while the cecum on the opposite side extends 0.63 mm anteriorly to the caudal margin of the vitellaria. In a third specimen, both cecum extend caudally to the vitellaria. The cecum on the vitellaria side exceeds the margin by 0.24 mm and the cecum on the opposite side by 0.15 mm. Thus, we do not consider extension of the cecum as a valid distinguishing characteristic between species of the genus *Athesmia*. In our study, all specimens collected from *C. brachyurus* were similar, with sub-equal cecum that both exceed the vitelline field, which differs from the identification key for the species of the genus *Athesmia* elaborated by Dronen (2014).

Travassos (1944), in his review of the family Dicrocoeliidae, when analyzing several characteristics of the parasites described in the genus *Athesmia*, considered it impossible to distinguish the different species based only on morphology. The only exemption are *A. wehri* and *A. parkeri*, because these two species have a relatively larger body than the others. The other species, according to Travassos (1944), were distinguished according to the geographic distribution and diversity of the hosts, which the author considered to be poor distinguishing characteristics. The same author considered only the following as valid species: *A. heterolechithodes* as a parasite of birds of the old world; *A. rudecta* as parasites of neotropic birds, considering *A. attilae*, *A. pricei* and *A. butensis* as synonyms thereof; *A. foxi* as a parasite of South American mammals; *A. wehri* as a parasite of neoartic birds with a relatively wide body; and *A. parkeri* as a parasite of Chiroptera, also with a wide body. In his review of the genus *Athesmia*, Travassos (1944) analyzed specimens of several hosts, concluding that they were all similar, but reiterated the species cited above, separated based on their hosts and distribution.

Byrd *et al*. (1967), after analyzing types, paratypes and additional specimens of *A. heterolechithodes*, *A. wehri* and *A. jollieie* deposited in the USNM Helminthological Collection, along with several

specimens studied by their group, concluded that the genus *Athesmia* is monotypic. *Athesmia heterolechithodes* is reported to infect terrestrial hosts, including desert environments, in addition to aquatic and semi-aquatic hosts (birds), suggesting that this parasite has no specificity for its definitive or for its intermediate hosts, which, according to these authors, may be an indication that this parasite infects both birds and mammals, with *A. heterolechithodes* being the unique species of the genus, in agreement with Freitas (1962).

Yamaguti (1971) recognized 11 species belonging to the genus *Athesmia*, with nine species being parasites of birds (*A. heterolechithodes*, *A. attilae*, *A. butensis*, *A. jolleie*, *A. kassimovi*, *A. pricei*, *A. reelfooti*, *A. rudecta* and *A. wehri*) and only two species being parasites of mammals (*A. foxi* and *A. parkeri*). Recently, another species of the genus *Athesmia* was described from a bird, *Rallus longirostris* (Gruiformes, Rallidae), named *A. ralli* Dronen, 2014, thus totaling 12 species of the genus *Athesmia*.

Dronen (2014) presented two identification keys for 11 species of the genus *Athesmia*, not including the species *A. rudecta*. In one of the keys, Dronen (2014) separates the species according to the body types described by him (atillae body type and heterolechithodes body type) and according second identification key, the author groups all species together, identifying them by morphological characteristics, mainly by the length and width of the eggs. According to several authors, the specimens collected by them in the same host may show differences in body size and internal organs (Faust, 1967; Byrd *et al*., 1967). However, Dronen (2014) considers that several authors (Freitas, 1963; Mettrick & Dunkley, 1968; Nasir & Díaz, 1971; Dronen *et al*., 2012) have noted that egg size is one of the least variable characteristics in groups of trematodes when it is considered to differentiate species.

Considering the great similarities between the species of the genus *Athesmia*, described by Travassos (1944), Freitas (1962), Faust (1967) and Byrd *et al*. (1967), who affirm that the species of the genus *Athesmia* have discrete morphological and morphometric differences; even when collected from the same hosts, molecular studies have become necessary to confirm the validity of the several species already described from this genus. However, based on the study of Travassos (1944) and Yamaguti (1971), who consider *A. foxi* and *A. parkeri* (wide body) the two parasitic species of mammals, we can infer that according to their morphology and morphometry, the specimens collected from *C. brachyurus* in the present study belong to the species *A. foxi*.

Research on the lesions caused by trematodes of the genus *Athesmia* is scarce. Kumar *et al*. (1980) described the histopathology of the liver of two monkeys, *Cebus albifrons*, infected with *A. foxi*, and reported that one of the monkeys had changes in tissue structure and necrosis, which were not observed in the liver of *C. brachyurus* from the present study. However, this monkey had a concomitant infection with pseudotuberculosis, which may have resulted in these necrotic changes. In the second monkey analyzed by Kumar *et al*. (1980) as well as in *C. brachyurus* in our study, the infection appears to have a proliferative and reactive aspect, but without necrosis such as those observed in the aforementioned monkey. All of the lesions observed in the liver of *C. brachyurus* are similar to those observed by Kumar *et al*. (1980) in *C. albifrons. E*xcept for the presence of evident hemorrhage in the hepatic parenchyma and the presence of macrophages in periductal cell infiltrates, which were not observed in the histological sections from recent study.

This is the first report of *A. foxi* parasitizing *C. brachyurus*, demonstrating that this parasite has no host specificity and is widely distributed. In addition, *A. foxi* lesions are similar across the various species of mammalian hosts. Thus, the present study shows important data related to a new parasitosis that affects *C. brachyurus*, an animal listed as near threatened in the IUCN Red List. The impact of this parasitosis on the health of these wild canids is unknown.

Conflict of interest

Authors state no conflict of interest.

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Research Note

Is there a host sex bias in intestinal nematode parasitism of the yellow-necked mouse (*Apodemus flavicollis*) at Obedska bara pond, Serbia?

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Summary

Fifty-one yellow-necked mice from the Obedska bara locality were analysed for the presence of intestinal nematode parasites in order to assert whether there was a host sex bias in infection. Previous research indicated that males would be the more infected sex, either due to the immunosuppressive effect of testosterone or their different allocation of resources towards immune defence. Quantitative infection parameters were compared between host sexes for all nematode species and nematodes in general. In addition, the influence of host sex, age, total body length, body mass and presence of other nematode species on parasite abundance was analysed. No statistically significant differences between males and females were noted for any of the studied quantitative parameters, leading to an absence of sex-biased parasitism in this study.
Keywords: nematodes; sex-biased parasitism; immunocompetence; host behaviour; Serbia

Introduction

Studies of sex-biased parasitism (SBP) date as far back as the beginning of the 20th century (Zuk & McKean, 1996), but the full extent of the phenomenon and the factors that underlie it remain open questions. In parasitological literature, studies generally report a male-biased infection (Poulin, 1996; Zuk & McKean, 1996; Krasnov *et al*., 2005). The two main groups of hypotheses explaining this refer to the immunosuppressive effect of the male sex hormone testosterone (Zuk & McKean, 1996; Schalk & Forbes, 1997; Zuk, 2009; Krasnov *et al*., 2012) and, more broadly, the differential effect of natural selection on males and females of the same host species (Zuk & McKean, 1996; Rolff, 2002; Nunn *et al*., 2009; Zuk, 2009). The main takeaway from both groups of hypotheses is that males trade off immunity for reproductive success, and thus become more susceptible to parasite infections.

The yellow-necked mouse, *Apodemus flavicollis*, is a rodent species that is a host of a number of ectoparasites and helminths. Experimental studies and mathematical modelling have shown that males play a crucial role in the transmission and maintenance of the nematode *Heligmosomoides polygyrus* in its populations (Ferrari *et al*., 2004, 2007), but to this date no prior study found evidence of sex-biased parasitism in this rodent species (Krasnov *et al*., 2012). The aim of the present study was to determine whether a host sex bias in intestinal nematode infection exists in a population of yellow-necked mice at Obedska bara pond. This preliminary analysis is the first study of sex-biased nematode parasitism to be carried out on this host species in Serbia.

Materials and Methods

A total of 51 individuals (30 males, 21 females) of the yellow-necked mouse were captured at Obedska bara locality, near the Sava river (44.746° N, 20.006° E) in October 2011. Obedska bara is a protected wetland and forest habitat situated on the Sava river, in the Srem (Syrmia) region of the northern autonomous

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province of Vojvodina, Serbia. Sampling of hosts was carried out in a mixed deciduous forest, consisting of trees such as English oak (*Quercus robur*), manna ash (*Fraxinus ornus*), black poplar (*Populus nigra*) and common hornbeam (*Carpinus betulus*). Trapping was carried out over three nights using 200 Longworth traps with bait and bedding supplied. Biometric data (body and tail length, body mass) were collected for all captured mice. In addition, host age was determined based on dry eye lens weight (Nabaglo & Pachinger, 1979).

All of the captured animals were sacrificed and kept in a freezer until dissection. Part of the intestinal tract (small intestine, caecum, colon and rectum) was removed from each animal and cut longitudinally in order to release its content into a Petri dish that was then emptied into a larger conical glass filled with tap water. Water was refilled and decanted in the conical glass until the supernatant was clear. All helminths were fished out under a stereomicroscope and conserved in 70 % ethanol. Identification was carried out based on morphological and morphometric characters using keys by Ryzhikov *et al*. (1979) and Genov (1984). The parasites were deposited in the collection of the Laboratory for animal ecology at the University of Novi Sad, protocol numbers 3391-3438, 3450-3451 and 3457.

Prevalence, mean and median intensity, and mean abundance for each nematode species and nematode parasites in total were calculated in accordance to Bush *et al*. (1997) for each host sex and for the total sample. Comparison of prevalence between host sexes was carried out via the exact unconditional test. Mean abundances and intensities were compared with a bootstrap test with 20000 replications, and median intensities were compared with Mood's median test. The null hypothesis was that there were no statistically significant differences between sexes with regards to the analysed parameters. All calculations and statistical tests were performed in Quantitative Parasitology software, version 3.0 (Rózsa *et al*., 2000).

Parasite abundance was further analysed by fitting abundance data in a generalized linear model (GLM). For the total sample, host age (with dry eye lens weight as a proxy), sex, total length and body mass were used as terms predicting the numerical response. Abundance data of each nematode species was also fitted, with host age, host sex, total length, body mass and abundances of the remaining species used as factors. The full models incorporated all of the terms and their two-way interactions. Factors and interactions below significance level (p=0.05) were excluded step by step until a minimal model was obtained. Calculations were performed in R software, version 3.2.1 (R Core Team, 2015) in standard packages.

Ethical Approval and/or Informed Consent

The research was conducted under permits issued by the Ministry of Natural Resources, Mining and Spatial Planning, Republic of Serbia (number: 353-03-250/2010-04). The mice were treated according to Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. All animal procedures were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade.

Results and Discussion

Of the fifty-one examined mice, forty (78.4 %) were infected with intestinal nematodes. Nematode prevalence was higher in male (83.3 %) than in female mice (71.4 %). Five roundworm species were recovered from the sample: *Aonchotheca annulosa*, *Heligmosomoides polygyrus*, *Syphacia frederici*, *S. stroma* and *Trichuris muris* (Table 1). Three of them were present in both host sexes, with *H. polygyrus* only recorded from a single female and *S. frederici* from a single male. Thus, the species richness of nematode parasitic community was identical, with four species found in each host sex. The average number of nematode species carried by infected individuals was 1.35. Infections with one nematode species were most common (70 % of infected hosts), with two (25 %) and three (5 %) species infections less frequently observed. While raw data seemed to suggest that males were more heavily infected (Table 2), statistical tests found no significant differences in infection between sexes. Comparisons of prevalence, mean intensity, mean abundance and median intensity between male and female total samples yielded no evidence of sex-biased parasitism. Furthermore, abundance data fitting showed no influence of host sex

Table 1. Parasitological parameters of infection for individual nematode species and intestinal nematodes as a group in the total sample of fifty-one mice from Obedska bara. 95 % confidence intervals in brackets, where applicable.

	I	P%	MI	MA
A. annulosa	12	23.5 (13.4 – 37.2)	8.1 (1.8 – 30.3)	1.9 (0.5 – 8.2)
H. polygyrus	1	2 (0.1 – 10.4)	1	0.02 (0 – 0.06)
S. frederici	1	2 (0.1 – 10.4)	13	0.3 (0 – 0.8)
S. stroma	30	58.8 (45.1 – 71.7)	29.2 (18.2 – 54.2)	17.2 (10.2 – 33.3)
T. muris	10	19.6 (10.5 – 33.2)	2.7 (1.5 – 4.9)	0.5 (0.2 – 1.2)
Nematodes	40	78.4 (64.8 – 87.9)	25.4 (16.4 – 44.8)	19.9 (12.4 – 35.5)

I – number of infected hosts, P% – prevalence, MI – mean intensity, MA – mean abundance

Table 2. Values of prevalence (P%), mean intensity (MI) and mean abundance (MA) for all nematode species and total intestinal nematodes in male and female yellow-necked mice from Obedska bara, Serbia. 95 % confidence intervals in brackets, where applicable.

	Males (30)			Females (21)		
	P%	MI	MA	P%	MI	MA
Aa	30 (16.3 – 48.3)	10.33 (1.7 – 34.1)	3.1 (0.5 – 12.7)	14.3 (4 – 35.4)	1.3 (1 – 1.7)	0.2 (0 – 0.5)
Hp	–	–	–	4.8 (0.3 – 23.3)	1	0.1 (0 – 0.14)
Sf	3.3 (0.2 – 17.7)	13	0.4 (0 – 1.3)	–	–	–
Ss	63.3 (45 – 78.6)	32.2 (16.8 – 73.1)	20.4 (10.1 – 47.1)	52.4 (30.5 – 72.4)	24.1 (11.9 – 42.6)	12.6 (5.5 – 25.8)
Tm	23.3 (11.2 – 41.6)	3.3 (1.6 – 5.9)	0.8 (0.3 – 1.8)	14.3 (4 – 35.4)	1.3 (1 – 1.7)	0.2 (0 – 0.5)
nem	83.3 (65.3 – 93.2)	29.6 (16.8 – 58.4)	24.7 (13.7 – 50.8)	71.4 (49.4 – 86.8)	18.3 (8.8 – 34.4)	13.1 (5.9 – 25.5)

Aa – Aonchotheca annulosa, *Hp – Heligmosomoides polygyrus*, *Sf – Syphacia frederici*, *Ss – S. stroma*, *Tm – Trichuris muris*, nem – nematodes

or any other analysed factor. The results of these analyses aren't shown but are available from the authors upon request.

Sex-biased parasitism in *A. flavicollis* has not been sufficiently addressed in the past and to the best of our knowledge no study that deals with it specifically exists for this rodent species. When such studies were performed on its close relative, the wood mouse (*A. sylvaticus*), the general conclusion was an absence of SBP (Goüy de Bellocq *et al*., 2003; Fuentes *et al*., 2004, 2007, 2010; Milazzo *et al*., 2010). Abu-Madi *et al*. (2000) state that intrinsic factors such as host sex, as well as host age, have a comparatively weak influence on the helminth fauna of *A. sylvaticus*. In light of previous data for the wood mouse, it may be possible that sex-biased parasitism as a whole does not occur, or is not common, in European *Apodemus* mice. However, an important conclusion reached by Ferrari *et al*. (2007) is that the sexes may differ in their relative contribution to parasite release and dispersal even in the absence of a bias in parasite levels, males and females having different roles in parasite dynamics. Males, due to being less immunocompetent and having wider ranges, generate a higher number of successful infective stages of parasites than females.

The present study found no evidence for sex-biased parasitism in the yellow-necked mouse at the Obedska bara locality, but it must be emphasized that this is a complicated and still incompletely understood phenomenon driven by a large assemblage of factors. More localities and more host/parasite combinations need to be studied, with the host sample structured with respect to age, habitat, trapping season and other relevant factors aside from sex. This preliminary study could possibly mark the beginning of such efforts, and lead to a better understanding of the mechanisms behind sex-biased parasitism not only in Serbia but also in general.

Conflict of Interest

Authors state no conflict of interest.

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Research Note

Infection status with plerocercoid of ligulid tapeworm in cyprinid fish from three lakes in Republic of Korea

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Article info

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Summary

We have investigated freshwater cyprinid fish for its current infection status with plerocercoid of ligulid tapeworm in the Republic of Korea. A total of 6,049 (517 Euiam Lake and 4,071 Hoengseong Lake in Gangweon-do and 1,461 Chungju Lake in Chungcheongbuk-do) fish were examined by dissecting the peritonial cavity between March 2015 and December 2016. Totally 45 (0.74 %) fish in of 5 (26.3 %) species (8 *Squalidus japonicus coreanus,* 6 *Squalidus gracilis majimae,* 7 *Opsariichthys uncirostris amurensis,* 15 *Zacco platypus* and 9 *Erythroculter erythropterus*) were infected with plerocercoids of ligulid tapeworm. The infection density with plerocercoids in *Erythroculter erythropterus* was 12 – 26 per fish infected in *Erythroculter erythropterus,* and 1 – 2 in other 4 fish species. The plerocercoid was ivory-white and 26.2 – 57.8 cm long. The prevalence value in this survey was 0.9 % (45/6,049). The genetic analysis in this study was conducted to identify plerocercoid species. Based on genetic analysis with data in GenBank, these plerocercoids were identified as the *L. intestinalis*.

Keywords: plerocercoid tapeworm; Chungju; Euiam and Hoengseong Lake

Introduction

The tapeworm *Ligula intestinalis* (Cestoda: Pseudophyllidea) is the most common species of the genus *Ligula* (Bloch, 1782). This organism, which has a three-host life cycle, infects a range of fresh water species in its plerocercoid stage, particularly members of the Cyprinidae, and as its second intermediate host and has a widespread distribution throughout the northern hemisphere (Bauer & Stolyarov, 1961; Dubinina, 1980; Brown *et al*., 2002; Hajirostamloo, 2009). A fish becomes infected through the ingestion of eating infected copepods. Once inside the fish, the tapeworm develops in the fish's body cavity into a larval stage known as a plerocercoid in the fish's body cavity, which goes on and to infect fish-eating birds such as herons and cormorants (Dubinina, 1980; Loot *et al*., 2001; Ergonul & Altindag, 2005). The parasites persist in the guts of birds for only a few days where they sexual maturity and to reproduce (Dubinina, 1980). As reported for different infected cyprinid species, regardless of sex, season and age, the gonads of infected fish remain immature and only early germ cell stages are present (Arme & Owen, 1968; Hoole, 1994; Olson *et al*., 2002). To date, only two papers have investigated *Ligula intestinalis* in Korea (Ryu & Lee, 1992; Sohn *et al*., 2016). However, a large-scale surveys about the infection status of *Ligula* plerocercoids in a variety of cyprinid fish species have not been conducted in Korea lakes. Many species of the genus *Ligula* are difficult to identify satisfactorily, at least if it is based on morphological characteristics alone. These organisms are flat, unsegmented and have a tapering anterior end with two bothridia pleurocercoids from different

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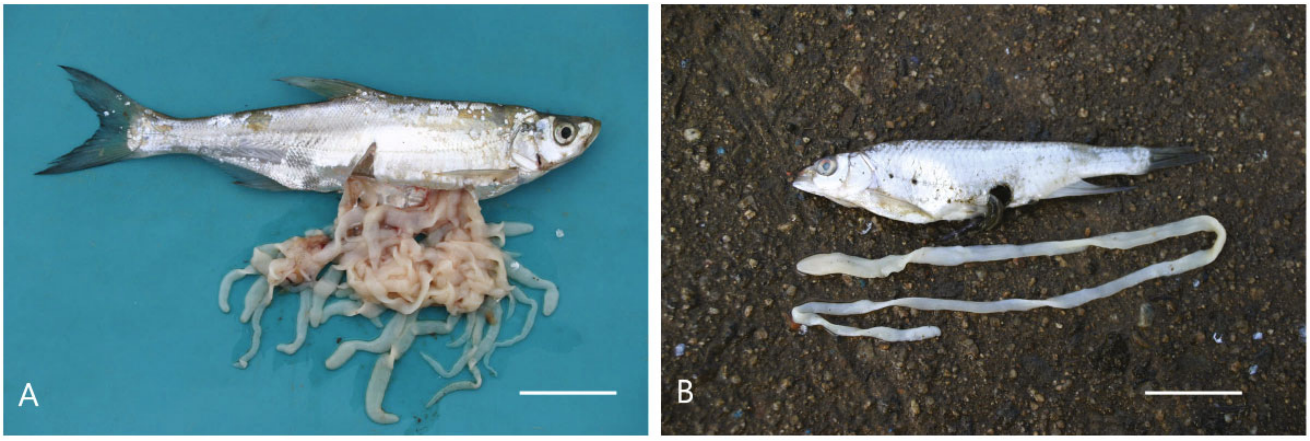

Fig. 1. An *Erythroculter erythropterus* with many plerocercoids of *Ligula intestinalis* from Chungju Lake(A) and an *Zacco platypus* with one plerocercoid from Hoengseong Lake (B). Scale bars are 5 cm.

host fish that vary in size from 10 – 100 cm in length, and 3 – 1.2 cm in width (Hajirostamloo, 2009). Recently, genetic analysis has been employed to distinguish *Ligula* species (Lee *et al.*, 2000; Li & Liao, 2003; Bouzid, 2008). Since *Ligula intestinalis* represents a complex of cryptic/sibling species, it is difficult to distinguish. Bouzid *et al*. (2008) and Štefka *et al*. (2009) have been reported that *Ligula intestinalis* seems to occur in Europe rather than in eastern Asia. The genetic analysis in this study was conducted to identify plerocercoid species. In the present study, we investigated the infection status of plerocercoid of the family Ligulidae from three lakes in Korea.

Materials and Methods

The infection status of freshwater cyprinid fish with *Ligula intestinalis* was examined in Chungju lake in Chungcheongbuk-do (do=Province), as well as Euiam and Hoengseong lakes in Gangwon-do, between March 2015 and December 2016. The fish were collected with the help of fisherman. We collected a total of 6,049 cyprinid fish belonging to 19 species from the three lakes once in every three months. The numbers and species of fish examined are shown in Table 1. Collected fish were transferred to the laboratory, classified to particular species and dissected to collect

Table 1. Infection status of cyprinid fish with plerocercoid of *L. intestinalis*, collected from Chungju, Euiam and Hoengseong lakes from Mar. 2015 to Dec. 2016.

Species	No. of fish collected from			Plerocercoid (mean no.)
	Euiam	Chungju	Hoengseong	
Carassius auratus	23	48	17	
C. cuvieri	2			
Cyprinus carpio	11	3		
Acheilognathus lanceolatus	25		8	
Acheilognathus yamatsutae	56	51		
Pseudorasbora parva	24	2	30	
Hemibarbus longirostris		3	4	
Pungtungia herzi	45	150	81	
S. nigripinnis.morii	28			
Squalidus japonicus coreanus	41	507(8*)		1 – 2
Squalidus gracilis majimae	22(6*)		27	1 – 2
Hemibarbus labeo	32	245		
H. longirostris	2			
M. yaluensis	3			
Pseudogobio esocinus	87	33	24	
Opsariichthys u. amurensis	21	69	1,816(7*)	1 – 2
Zacco platypus	95		2,064(15*)	1 – 2
Erythroculter erythropterus		311(9*)		12 – 26
Hemiculter leucisculus		39		
Total number of collected fish	517	1,461	4,071	

*: No. of infected fish with plerocercoid of *Ligula intestinalis*

Table 2. Number of infected fish with plerocercoid of *L. intestinalis*, collected from Chungju, Euiam and Hoengseong lakes from Mar. 2015 to Dec. 2016.

Date of fish collection	Number of infected fish			
	Euiam Lake	Hoengseong Lake	Chungju Lake	Total
20 Mar. 2015	1	1	1	3
21 June	1	0	1	2
14 Sep.	1	3	2	6
8 Dec.	0	8	3	11
19 Mar.	2	2	1	5
7 June	0	0	1	1
28 Sep.	0	2	3	5
3 Dec.	1	6	5	12
Total	6	22	17	45

the parasites. Plerocercoid present in the peritoneal cavity were counted, and identified using the characteristics suitable for species identification according to the criteria of Dubinina (Dubinina, 1980) and Bykhovskaya-Pavlovskaya et al. (Bykhovskaya-Pavlovskaya, 1962). Plerocercoid samples were preserved by either placing them in absolute ethanol or freezing them at -80 °C. Moreover, genetic analysis was conducted to identify plerocercoid species. Mitochondrial cytochrome *c* oxidase subunit I (*Cox 1*) and cytochrome *b* (*Cytb*) genes were used as targets (Bouzid, 2008). A total of 20 plerocercoid samples from five fish species were examined and compared with the GenBank-registered *Ligula* species.

Results

A total of 6,049 fish (517 from Euiam Lake, 1,461 from Chungju Lake and 4,071 from Hoengseong Lake) belonging to 19 species were examined by dissecting the peritoneal cavity in the present study (Table 1). Five of the 19 species of cyprinids fishes collected in three lakes were infected with plerocercoid of ligulid tapeworm. The infection status according to the fish species and surveyed localities is particularized in Table 1. Overall, totally 4,973 fish from five infected species in three lakes were investigated and the infection prevalence (0.9 %, 45/6.049) ranging from 0.2 % in January – July to 0.7 % in September – November (Table 2) was very low. The positive rates of fish with plerocercoids were as follows: 1.5 % (8) *Squalidus japonicus coreanus,* 12 % (6) *Squalidus gracilis majimae,* 0.4 % (7) *Opsariichthys uncirostric amurensis,* 0.7 % (15) *Zacco platypus* and 2.9 % (9) *Erythroculter erythropterus.* The plerocercoid was ivory-white and 26.2 – 57.8 cm long (Fig. 1). In *Erythroculter erythropterus*, the number of plerocercoid in the peritoneal cavity was 12 – 26, while the other four species had 1 – 2 plerocercoid in the peritoneal cavity. To obtain definitive information regarding the taxonomy of *Ligula* plerocercoid, the partial nucleotide sequence of mitochondrial *Cox 1* and *Cytb* genes were performed. The *Cox 1* (395 bp) and *Cytb* (405 bp) sequences obtained upon gene analysis were compared with GenBank data (Accession number; KY321843.1, KY321844.1, AF153910.1, EU241317.1, EU241316.1, EU241315.1, & EU241218.1, EU241217.1, EU241216.1, EU241215.1), and a 0.1 % genetic difference (2 – 4 bp) from *Ligula intestinalis* was observed. Based on comparison of the results of genetic analysis with data in GenBank, this plerocercoid was identified as *L. intestinalis*.

Discussion

It is well known that the tapeworm *Ligula intestinalis* infects many different species of freshwater fish, but primarily infects cyprinids (Sweeting, 1977; Barus & Prokes, 2002; Ergonul & Altindag, 2005). A fish becomes infected through the ingestion of infected copepods, after which the tapeworm develops into a larval stage called a plerocercoid in the fish's body cavity. The definitive hosts are ichthyophagous predatory birds such as herons and cormorants, in which *L. intestinalis* reaches sexual maturity. Parasite eggs are then released into the water with bird feces (Hoole, 1994). To date, a total of four species of freshwater fishes, *Zacco platypus, Carassius auratus, Hemiculter bleekeri* and *Chanodichthys erythropterus,* have been reported as the second intermediate hosts of *Ligula intestinalis* in Korea (Ryu & Lee, 1992; Sohn *et al*., 2016). *Ligula* pleurocercoids show very limited structural differentiation. Specifically, they are flat, unsegmented and have a tapering anterior end with two bothridia plerocercoids from different host fish that vary in size. Early accounts concerning the genus *Ligula* were reviewed by Cooper (1918), who recognized only the single species of *L. intestinalis*, and listed 63 species of the host fish from many different families (Arme & Owen, 1968; Hajirostamloo, 2009). Bykhovskaya-Pavlovskaya et al. suggested that at least two genera (*Digramma* and *Ligula*) and five species, including *L. intestinalis*, are involved and that the plerocercoid of each species is restricted to a relatively narrow range of host species. However, Luo et al (2003) compared *Digramma* and *Ligula* specimens based on ITS and the 5' end of 28S rDNA sequences and found that low level nucleotide variation between the two genera may imply that cestodes in the genus *Digramma* are paraphyletic to the *Ligula* genus, and that *Digramma* is a synonym of *Ligula*. To obtain definitive information regarding the taxonomy of *Ligula* plerocercoid obtained in this survey, we also analyzed the partial nucleotide sequence of mitochondrial *Cox 1* and *Cytb* genes. Based on comparison of

the data obtained by genetic analysis to that in GenBank, these plerocercoids were identified as *L. intestinalis*.

The cyprinid fish that serve as the second intermediate hosts of this tapeworm have been reported in a variety of locations worldwide (*Alburnus filippi, Alburnoides bipunctatus, Capoeta capoeta, Cyprinus cpito*, *Gobio gobio, Salmo trutta*, *Scardinius erythrophthalmus, Leuciscus cephalus*, *Leuciscus leuciscus*, *Phoxinus phoxinus*, *Rutilus rutilus*, *Hemiculter bleekeri*, *Barbus* spp. and *Blicca bjoerkna*) (Arme & Owen, 1968; Charles & Orr, 1968; Hoole, 1994; Loot, 2001; Britton, 2009; Hajirostamloo, 2009; . Vanacker, 2012; Tizie *et al*., 2014; Sohn *et al*., 2016). The infected cyprinid fish confirmed in this study were *Squalidus gracilis majimae, Opsariichthys uncirostric amurensis, Zacco platypus, Squalidus japonicus coreanus* and *Erythroculter erythropterus*. The preponderance of infection in Korean cyprinids seems to be related to the widespread distribution and abundance of these fish. Although typically reported from cyprinid fish, *L. intestinalis* has been shown to utilize a broad range of hosts, including other fish families such as Catostomidae, Salmonidae or Galaxiidae (Dubinina, 1980; Groves & Shields, 2001; Barus & Prokes, 2002). Therefore, other freshwater families should be investigated in the future. *Ligula intestinalis* infections reportedly tend to occur between July and December (Loot *et al*., 2001). Infected fish in this study were found mainly among those caught in summer to winter. Thus, during autumn, *L. intestinalis* grows markedly in the host body cavity, and plerocercoids may reach a size between 10 and 30 cm long (Hoole, 1994). Since the worms in the bird host acquire maturity in 3 – 5 days in the bird host, infected piscivorous birds are rarely observed under natural conditions (Loot *et al.,* 2001). Thus, it is difficult to determine which bird species in the lakes play a key role in the transmission of *L. intestinalis* in the lakes. Accordingly, future studies investigating what kinds of bird are transmitting *L. intestinalis* in Korea are warranted.

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Research Note

First report of entomopathogenic nematode *Steinernema feltiae* (Rhabditida: Steinernematidae) from Croatia

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Article info

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Summary

A survey of entomopathogenic nematodes was conducted in Croatia between 2016 and 2017. The steinernematids were recovered in two out of 100 soil samples from agricultural land characterized as loamy soils with acidic reaction. Molecular and morphological identification was used to distinguish the nematodes. The isolates were identified as two different strains conspecific with *Steinernema feltiae.* The variations in morphometrical characteristics of infective juveniles (IJs) and males were observed among Croatian strains and with the original description. The analysis of ITS region revealed the greatest similarity of Croatian strains with Slovenian B30 and English A2 strains, which together comprised a monophyletic group in evolutionary analysis. This is the first record of steinernematids, namely *S. feltiae* in Croatia**.**

Keywords: *Steinernema feltiae;* strain; Croatia; morphological variations*;* survey

Introduction

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* Travassos and *Heterorhabditis* Poinar are effective biological control agents against wide range of insect pests (Lacey & Georgis, 2012). Entomopathogenic nematodes actively seek for their host and leave no ecological footprint. These are the main advantages of EPNs as biocontrol agents over chemical pesticides (Kaya *et al*., 2006). The infective or third stage juvenile (IJs) carry in its intestine symbiotic bacteria which are released into the insect hemocoel once the nematode is inside the host. The bacteria produce highly virulent insecticidal toxins which kill the host within 24 to 72 h by septicaemia. Bacteria of the genus *Xenorhabdus* Boemare, Akhurts and Mourant are associated with steinernematids, while bacterial species of genus *Photorhabdus* Thomas and Poinar are symbionts *of* heterorhabditids (Koppenhöfer, 2007). They are globally distributed, but their species biodiversity and distribution is still unrevealed in some countries and regions (Adams *et al*., 2006). In Southeastern Europe there are records of EPNs from several countries only: Slovenia (Laznik *et al*., 2009), Bosnia and Herzegovina (Iqbal & Ehlers, 2016), Serbia (Tallosi *et al*., 1995), Bulgaria (Shishiniova *et al*., 1997), Greece (Menti *et al*., 1997), and Albania (Tarasco & Poliseno, 2005). An increasing trend in research and commercialization of EPNs has been observed throughout the world (Kaya *et al*., 2006). Primarily due to removal of chemical pesticides from the market due to the toxicological issues and need for the efficient biocontrol agent. Accordingly, it is important to isolate nematodes from different geographical regions, and also to evaluate and compare native with commercial strains for their potential in biocontrol programs in specific area (Laznik *et al*., 2010). Species from different regions are locally adapted and potentially different in terms of reproduction, infectivity, host range, and conditions for survival which have to be documented (Hazir *et al*., 2003). The aim of this study was to conduct survey in Croatia and report indigenous EPNs species.

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Material and Methods

Soil samples were collected in the eastern part of continental Croatia in 2016 and 2017. Sampling was conducted during spring and autumn months from diverse habitats. In total, 100 soil samples were collected. Soil characteristics and habitats of the positive sampling sites are presented in Table 1. Soil was collected from the upper 3 – 30 cm layer, and within each site a sample consisted of 1 kg of soil were randomly taken from five subsamples. Samples were kept in polyethylene bags and refrigerated until extraction. The samples were processed within two days upon collection. Each sample was thoroughly mixed and subsample of soil was taken from each sample, for analyses of soil type, organic matter content and pH. The insect baiting technique was used as described by Kaya and Stock (1997) with modification of insect host. *Achroia grisella* Fab., the lesser wax worm was used as baiting insect. The lesser wax moth larvae were placed in pierced Eppendorf tubes in each soil sample. In total, each soil sample received five baited tubes. The baited samples were kept in dark at room temperature (20 – 22 °C) for a period of 14 days. At two days interval, insect cadavers were collected and placed in a modified White trap to harvest emerging nematodes. The harvested IJs were stored in culture flasks in saline solution (M9 buffer) at 4 °C in refrigerator. In order to establish culture, ten live larvae of the lesser wax moth were placed in Petri dish lined with filter paper and infected with 50 IJs per insect larvae from each positive sample. The Petri dishes were incubated at room temperature in dark. Nematode progeny was used for molecular and morphologically based identification (Kaya & Stock, 1997). Twenty individuals of first generation males and IJs were fixed and transferred to anhydrous glycerin. Nematodes were examined under an Olympus BX50 (Japan) microscope equipped with differential interference contrast optics and digital image software (Olympus LCmicro 2.1, Japan). The morphometrics of nematode body measurements are presented in Table 2. Polymerase chain reaction (PCR) was performed to multiply ITS (internal transcribed spacer) region from genomic DNA extracted from single individual using primers TW81 and AB28 after Hominick *et al*. (1997). The PCR products were re-isolated from 1 % TAE-buffered agarose gel using E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, USA). Reisolated sample was se-

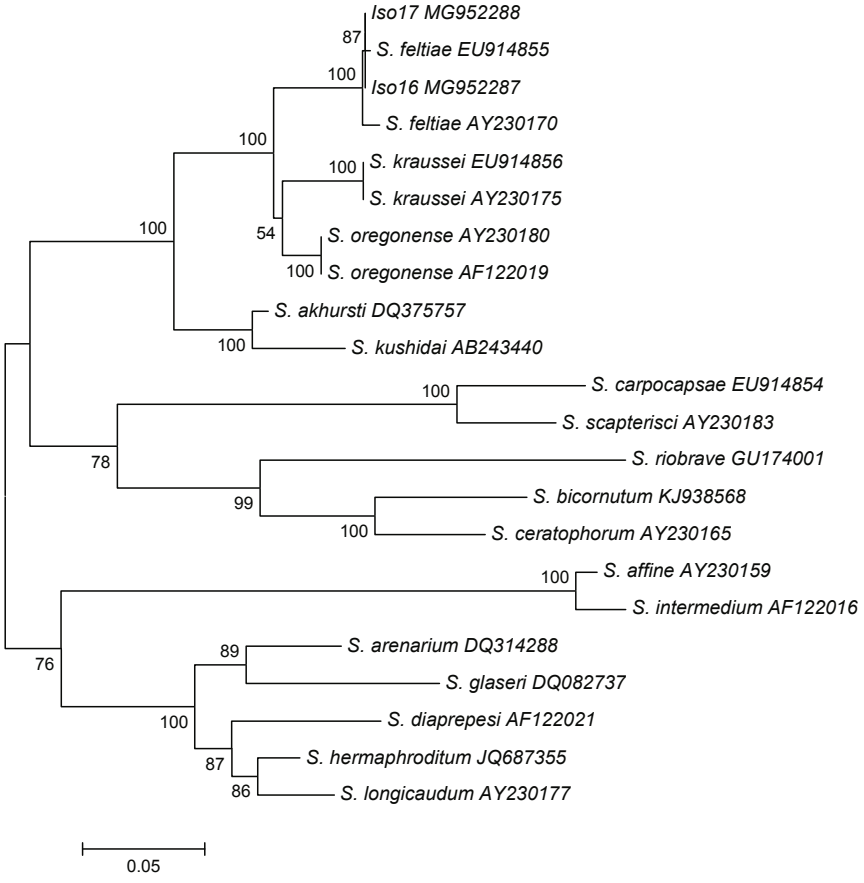

Fig. 1. Evolutionary relationships of new Croatian EPN isolates (ISO16 and ISO17). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

Table 1. Locality, soil characteristics, habitats and sampling time of the positive sites of *Steinernema feltiae*.

Strain	Location	Coordinates	Soil type	Organic matter (%)	pH (H_2O)	Habitat	Vegetation	Sampling date
ISO16	Arduševac	45°18'46.58"N 18°30'58.68"E	Loam	2.10	5.26	Agricultural	Potato	October 2016
ISO17	Arduševac	45°8'45.89"N 18°30'53.19"E	Loam	3.69	5.95	Agricultural	Fallow	

quenced in the laboratory of Agricultural Biotechnology Centre in Gödöllő, Hungary. Sample DNA sequences represented species of all the five main clades inside the *Steinernema* genus (Spiridonov *et al*., 2004) were used to phylogenetic analysis. ITS1, IT2 and 5.8S rRNA gene sequences were aligned using CLUSTAL_X 2.0 (Larkin *et al*., 2007). The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 495 positions in the final dataset. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura *et al*., 2011).

Ethical Approval and/or Informed Consent

This article does not contain any studies with human participants or animals by any of the authors.

Results

The steinernematids were recovered in two out of 100 soil samples, in fall from agricultural land used for growing potatoes and fallow, respectively. Soil characteristics of the positive sites are classified as loam with slightly acidic reaction, pH varied from 5.26 to 5.96. There was no obvious influence of organic matter content in recovery of steinernematids, since the nematodes were extracted from soils with differing organic matter content (2.10 % and 3.69 %), (Table 1).

Morphometric data obtained for males and IJs showed that nematodes belong to the family Steinernematidae, and both isolates (ISO16 and ISO17) are conspecific with *Steinernema feltiae* Filipjev 1934. The Croatian strains of *S. feltiae* are morphologically similar to known species, with variations of IJs of ISO17 strain. Compared to the ISO16 strain and known species, IJs of ISO17 appeared smaller for all measured characteristics. Values obtained for first generation males lie with the ranges measured of previously described *S. feltiae* (Poinar, 1990). The morphological characteristics presented in Table 2 are considered as the most reliable for *S. feltiae* (Adams & Nguyen, 2002).

The phylogenetic relationship of the studied isolates of *S. feltiae* and homologous sequences of the same genus from the GenBank is presented in Figure 1. The phylogenetic tree revealed five major clades. Both Croatian isolates were grouped with two strains *S. feltiae*. Multiple sequence alignment of the Croatian strains *S. feltiae* with sequences of other populations of the same species showed 99 % similarity with Slovenian strain *S. feltiae* B30 (GenBank EU914855) (Laznik *et al*., 2009). Overall, five major clades are supported by a high bootstrap value ranging from 99 to 100 %. The sequences were deposited in GenBank under accession numbers MG952287 (ISO16) and MG952288 (ISO17).

Discussion

This is the first record of EPNs of family Steinernematidae, namely *S. feltiae* in Croatia. The efficiency of recovery of steinernematids in our study was 2 %, similarly to earlier reports by Laznik *et al*. (2009). *Steinernema feltiae* was recovered from agricultural fields only, and both positive samples were taken at close locations. Depending on the climate and the region, the members of family Steinernematidae show prevalence for specific habitats. Results

Table 2. Comparative morphometric data of *Steinernema feltiae* (Croatian isolates and known species). All measurements represent mean and range in µm.

Isolate	IJs				Male		
	L[a]	EP	T	E%	SL	D%	MUC
ISO16	842 (733 – 929)	61 (50 – 69)	81 (72 – 90)	76 (65 – 86)	69 (59 – 77)	62 (48 – 70)	P
ISO17	801 (699 – 937)	60 (47 – 71)	77 (69 – 86)	76 (68 – 88)	70 (61 – 76)	62 (50 – 71)	P
S. feltiae[b]	849 (736 – 950)	62 (53 – 67)	81 (70 – 92)	78 (69 – 86)	70 (65 – 77)	60	P

[a]L = body length, EP = distance from anterior end to excretory pore, T = tail length, SL = spicule length, D% = EP/oesophagus length × 100, E% = EP/T × 100, MUC = mucron, P = present.
[b]After Poinar (1990)

from surveys in the UK, the Netherlands and Germany reveal that *S. feltiae* prefers fields and grassland (Sturhan & Liskova, 1999), and our findings support these results. However, *S. feltiae* has been recovered from the forest biotopes often (Spiridonov and Moens, 1999; Tarasco *et al*., 2015). Soil properties such as moisture level, pH, organic matter content, texture and others, affect EPNs dispersal and potential to find host (Stuart *et al.,* 2015). Entomopathogenic nematodes move more freely and find host in lighter soils. Their locomotion decreases with smaller size of soil particles (Glazer, 2002). However, for recovery of steinernematids, soil type is considered of minor importance (Sturhan, 1999). We recovered *S. feltiae* from loamy soils which contain balanced mixture of light (sand) and heavy (clay) particles with acidic reaction, similar to the results of survey in California (Stock *et al*., 1999). Koppenhöfer and Fuzzy (2006) found that infectivity of tested heterorhabditids and steinernematid species is lower in acidic conditions, while preference for soil texture was species specific. Organic matter content did not influence recovery of *S. feltiae* in our study. Our results support findings of Hazir *et al*. (2003) who also reported natural occurrence of *S. feltiae* in soils differing in organic matter content.

The Croatian *Steinernema* isolates (ISO16 and ISO17) were identified as strains *S. feltiae*. However the variability in morphometrics was observed within the strains and with the original descriptions (Poinar, 1990). Body length and other characters of IJs *S. feltiae* ISO17 were comparatively smaller, while strain ISO16, morphologically resembles more the originally described *S. feltiae* than Croatian ISO17 strain. Morphological and morphometric variations of EPNs can be host (Campos-Hererra *et al*., 2006) or temperature induced (Hazir *et al*., 2001). In this study we used different host (the lesser instead of greater wax moth) and this could be the reason of the observed variability with the original described by Poinar (1990). Furthermore, it has been suggested that IJs body length is longest when EPNs are reared at 8 °C and becoming shorter if reared at room temperature conditions (Hazir *et al*., 2001). The Croatian strains were reared at room temperature, and this could be another reason for shorter morphometric values of ISO17. The difference among the Croatian strains can be due to the intraspecific variability (Stock *et al*., 1999). Furthermore, the morphological differences can be expected when populations of different geographic origins are compared (Stock *et al*., 2000). According to the molecular characterization, both Croatian strains are closest to two European isolates, Slovenian strain *S. feltiae* B30 (GenBank EU914855; Laznik *et al*., 2009) and English strain *S. feltiae* A2 (GenBank AY230170; Reid & Hominick, 1993) (Fig.1). These four strains of *S. feltiae* comprise a monophyletic group by analysis of the ITS region. Groups of closely related isolates of steinernematids are often determined by specific geographical area of distribution (Spiridonov *et al*., 2004). From ITS region of Slovenian strain *S. feltiae* B30, both Croatian strains differ by 33 (ISO16) and 34 bp (ISO17). The strains (ISO16 and ISO17) differ from each other by 1 bp. We expected greater species diversity and more positive sites for steinernematids. Since this genus has more described species, and generally are recovered more often than heterorhabditids. *Steinernema feltiae* are found globally in all terrestrial habitats. However, they have a wider distribution in temperate regions (Adams *et al*., 2006). Our results extend the range of geographical distribution of *S. feltiae*, indicating the plasticity of the species to adapt to the conditions of southeastern parts of Europe. The Croatian strains *S. feltiae* should be tested for pathogenicity and possibly included in future biological or integrated pest management programs in Croatia and other countries with similar climates. This indicates need for further research on EPNs biodiversity in Croatia and host ranges.

Conflict of Interest

Authors state no conflict of interest.

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