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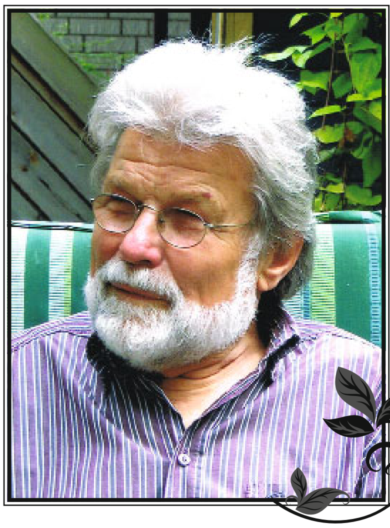
EDITORIAL

Dieter Sturhan Dr. Rer. Nat. (1936 – 2017)



Dr. Dieter Sturhan was born on 30th September 1936 in Meerbeck in Lower Saxony, and died on 29th November 2017 in Muenster. He studied zoology, botany, and geography at Universities of Kiel, Munich, and Erlangen. He acquired his PhD. in 1962, and in the same year started working at the Institute for Nematology of the Biological Centre for Agriculture and Forestry in Muenster where he worked with small breaks until his retirement. His work abroad was quite extensive, he acted long-term as a consultant for nematology in German government's projects in several foreign countries. Within international scientific cooperation with foreign countries, he worked on nematological research projects with many nematologists including us in Slovakia.

In his life-long nematological research, Dr. Sturhan focused on diagnostics, taxonomy, biology, ecology and geographic expansion of soil and plant nematodes, in particular of phytopathologically important parasitic plant nematodes. These included mainly plant nematodes of the Longidoridae, Trichodoridae, Heteroderidae, Meloidogynidae, and Aphelenchoididae families. His extensive publishing work reflects the preciseness and strenuousness of Dr. Sturhan's scientific work, and it is a source of knowledge for generations of nematologists around the world.

Besides his research, Dr. Dieter Sturhan worked in several nematological, zoological, ornithological, and phytopathological societies of the world. Since 1974, he worked as a German correspondent for the European Society of Nematologists, and he was a member of editorial boards of several nematological magazines around the world. At home in Germany, he regularly organized and conducted annual courses of practical identification of parasitic plant nematodes for plant protection workers, taught applied zoology at the University of Muenster, and worked as the chairman of Nematological Society at the German Phytomedical Society.

Apart from scientific work, the character of Dieter Sturhan must be highlighted. He considered his co-workers not only as colleagues – nematologists but also as close friends, often family friends. It made no difference to him if they were from Eastern or Western Europe, Asia, or America. For many years, Dieter Sturhan committed himself in helping the homeless, immigrants from south-eastern Asia, and was part of the German Peace Movement.

With the passing of Dr. Dieter Sturhan we lose an exceptional scientist, nematologist colleague, and a precious friend.

Marta Lišková, Zuzana Vasilková

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New data on the morphology and phylogenetic connections of *Postlepidapedon opisthobifurcatum* (Trematoda, Lepocreadioidea: Lepidapedidae), a parasite of Antarctic and sub-Antarctic fishes

S. G. SOKOLOV[1], F. K. KHASANOV[1], I. I. GORDEEV[2,3,*]

[1]Center of Parasitology, A.N. Severtsov Institute of Ecology and Evolution RAS, Leninskij prospect, 33, Moscow, 119071, Russia; [2]Lomonosov Moscow State University, Leninskie Gory, 1, Moscow, 119234, Russia, *E-mail: *gordeev_ilya@bk.ru*; [3]Russian Federal Research Institute of Fisheries and Oceanography, V. Krasnoselskaya Str., 17, Moscow, 107140, Russia

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Summary

The trematode *Postlepidapedon opisthobifurcatum* (Zdzitowiecki, 1990) is a common intestinal parasite of the gadiform fishes of the Southern Ocean. In this work, we supplement the description of the species with the anatomy of the terminal part of the reproductive system and with molecular data. The male terminal genitalia are characterised by the presence of the external seminal vesicle and cirrus-sac. The external seminal vesicle is surrounded by aciniform groups of outer prostatic cells. Groups of outer prostatic cells and proximal parts of their ducts are associated with a thin-walled membrane that is connected to the proximal edge of the cirrus-sac. The cirrus-sac is claviform, with a long proximal part accommodating the tubular, thin-walled internal seminal vesicle and ducts of outer prostatic cells. The female terminal genitalia are represented by a thick-walled metraterm, which is surrounded by aciniform groups of glandular cells. Phylogenetic analysis based on 28S rDNA partial sequences data placed *P. opisthobifurcatum* into the monophyletic group Lepidapedidae, including the species *Myzoxenus insolens* (Crowcroft, 1945), *Intusatrium robustum* Durio et Manter, 1968, and *Postlepidapedon uberis* Bray, Cribb et Barker, 1997. However, we were unable to detect direct phylogenetic connections between *P. opisthobifurcatum* and *P. uberis*.
Keywords: Trematoda; *Postlepidapedon opisthobifurcatum*; Lepidapedidae

Introduction

The trematode *Postlepidapedon opisthobifurcatum* (Zdzitowiecki, 1990) Zdzitowiecki, 1993 is one of the common intestinal parasites of Antarctic and sub-Antarctic fishes of the families Muraenolepididae and Macrouridae (Zdzitowiecki, 1990; Zdzitowiecki & Cielecka, 1997; Walter et al., 2002; Sokolov & Gordeev, 2013; 2015; Gordeev & Sokolov, 2017). This species was originally included in the genus *Neolepidapedon* Manter, 1954 (see Zdzitowiecki, 1990). Zdzitowiecki (1993) subsequently erected a new genus, *Postlepidapedon*, based on the morphology of the cirrus-sac (presence of elongated, thin-walled internal seminal vesicle and narrow, long ejaculatory duct), the position of the intestinal bifurcation, and a number of other characters to accommodate *Neolepidapedon opisthobifurcatus* Zdzitowiecki, 1990. Other than the type species (*P. opisthobifurcatum*), this genus currently includes five other congeners, described from perciform fishes from the waters off Australia, New Caledonia, and the Philippines (Bray *et al.*, 1997; Bray & Cribb, 2001; Machida, 2004). Bray & Cribb (2012) placed the genus *Postlepidapedon* into the family Lepidapedidae.

The aim of the present study is to describe in more detail the morphology of the terminal part of reproductive system of adult *P. opisthobifurcatum* and to define the phylogenetic position of this species based on molecular data.

* – corresponding author

Materials and Methods

Specimen collection and morphological study

The digeneans were collected during parasitological examination of specimens of *Muraenolepis marmorata* Günther, 1880 (Gadiformes, Muraenolepididae) caught on 18 March 2013 in the central part of the Ross Sea (75°48S 172°48W). All hosts (length 49 – 55 cm, weight 0.9 – 1.4 kg) were caught by the fishing vessel *Yantar-35* while it was fishing for the toothfish *Dissostichus* spp. at depths ranging from 962 m to 1228 m using bottom longline fishing gear (autoline) ''Mustad'' (Petrov et al., 2014) inside the Convention for the Conservation of Marine Living Resources (CCAMLR) area. The worms collected for morphological study were fixed in 70 % ethanol under a cover glass without additional pressure and stained with acetocarmine. Trematode species were identified using the publications of Zdzitowiecki (1990; 1993) and Zdzitowiecki & Cielecka (1997). The drawing and dimensions of *P. opisthobifurcatum* from our collection are given in Sokolov & Gordeev (2013).

The description of the terminal part of the reproductive system is based on the study of isolated organs (from 20 specimens) extracted from the bodies using needles. The worms used in the phylogenetic analysis were fixed in 95 % ethanol. Voucher specimens of the studied species were deposited in the Museum of Helminthological Collections, Centre for Parasitology, A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, Russia (IPEE RAS).

DNA extraction, amplification, sequencing, and phylogenetic analysis

Genomic DNA was extracted from single specimens of adult worms following the protocol used by Tkach *et al.* (1999). Nuclear 28S rDNA partial fragment, including D1-D3 domains, was amplified using a polymerase chain reaction by the following primers: DIG12 (5'- AAG CAT ATC ACT AAG CGG-3') and 1500R (5'- GCT ATC CTG AGG GAA ACT TCG-3') (Tkach *et al.*, 2003). The initial polymerase chain reaction was carried out in a total volume of 25 µl containing 0.25 mM of each primer pair, 5 µl DNA in water, 1× Phusion polymerase buffer, 2.5 mM dNTP, and 1 unit of Phusion High-Fidelity DNA Polymerase (New England Biolabs, UK). The amplification of a 1330-bp fragment of 28S rDNA was performed in a GeneAmp 9700 (Applied Biosystems) with a 1-min denaturation hold at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 60 s at 72 °C; followed by a 2-min extension hold at 72 °C. Negative and positive controls, using both primers, were used. The PCR products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit, as recommended by the manufacturer, with the internal sequencing primers 300F (5'-CAA GTA CCG TGA GGG AAA GTT G-3') , ECD2 (5'–CTT GGT CCG TGT TTC AAG ACG GG–3'), 900F (5'-CCG TCT TGA AAC ACG GAC CAA G-3'), and 1200R (Tkach *et al.*, 2003). The sequences obtained have been submitted to NCBI GenBank (Table 1).

Phylogenetic relationships were performed using our data and nucleotide sequences of 28S rDNA of lepidapedids from the NCBI GenBank database (Table 1). Due to the limited data on trematodes of the family Lepidapedidae deposited in the GenBank, we also used an original sequence of *Muraenolepitrema magnatestis* Gaevskaya et Rodjuk, 1988 collected from the same host and in the same region as *P. opisthobifurcatum* (see above, and Sokolov & Gordeev, 2015). Representatives of the families Enenteridae and Gyliauchenidae (Table 1) were selected as the outgroup based on their phylogenetic position relative to the family Lepidapedidae (see Bray & Cribb, 2012). Most of the 28S rDNA sequences of lepidapedids deposited into NCBI GenBank database are about 900 bp long. In this regard, two variants of the tree are given in this paper. The first is built based on 900 bp, and includes the maximum number of species of the Lepidapedidae deposited in GenBank. The second one is built with 1230 bp alignment length and includes a limited number of species. Initially, the sequences were aligned with the aid of ClustalX using default parameters (Jeanmougin *et al.*, 1998), and then they were refined by estimating the number of variable sites and sequence differences using MEGA 6.0 (Tamura *et al.*, 2013). Phylogenetic analysis of the nucleotide sequences was performed using Bayesian inference implemented in MrBayes v.3.2.6 on CIPRES portal (Miller *et al.*, 2010). The analysis was conducted using the GTR+I+G model, where ngen was set to 5×10^6, with two runs each containing four simultaneous Markov ChainMonte Carlo (MCMC) chains and every 10000th tree saved. An evolutionary model for the Bayesian inference analysis was selected using MEGA v.7.0.21 (Kumar *et al.*, 2016). Samples of substitution model parameters and tree and branch lengths were summarised using the parameters ''sump burnin = 0.25'' and ''sumt burnin = 0.25''. The significance of the phylogenetic relationships was estimated by posterior probabilities (Huelsenbeck *et al.*, 2001).

Ethical Approval and/or Informed Consent

The conducted research is not related to either human or animals use. Informed consent has been obtained from all individuals included in this study.

Results

Morphology of the terminal part of the reproductive system

The male terminal genitalia are represented by the external seminal vesicle, cirrus-sac, and complex of outer prostatic cells (Fig. 1). The vas deferens is absent; vasa efferentia is joined directly to the external seminal vesicle. The external seminal vesicle is large, 1.5 – 2 times longer than the cirrus-sac, convoluted, and surrounded by aciniform groups of outer prostatic cells radiating into the parenchyma. Groups of outer prostatic cells and proximal parts of their ducts are covered with a thin-walled open-ended membrane. The membrane is divided into two sheets – dorsal and ventral.

Table 1. List of taxa, incorporated into molecular analysis.

Species	Family	Reference	GenBank accession number
Bulbocirrus aulostomi Yamaguti, 1965	Lepidapedidae	Bray *et al.* (2009)	FJ788470
Intusatrium robustum Durio et Manter, 1968	Lepidapedidae	Bray *et al.* (2009)	FJ788481
Lepidapedon arlenae Bray et Gibson, 1995	Lepidapedidae	Bray *et al.* (1999)	AJ405262
Lepidapedon beveridgei Campbell et Bray, 1993	Lepidapedidae	Bray *et al.* (1999)	AJ405263
Lepidapedon desclersae Bray et Gibson, 1995	Lepidapedidae	Bray *et al.* (1999)	AJ405264
Lepidapedon discoveryi Bray et Gibson, 1995	Lepidapedidae	Bray *et al.* (1999)	AJ405265
Lepidapedon elongatum (Lebour, 1908)	Lepidapedidae	Bray *et al.* (1999)	AJ405266
Lepidapedon gaevskayae Campbell et Bray, 1993	Lepidapedidae	Bray *et al.* (1999)	AJ405267
Lepidapedon rachion (Cobbold, 1858)	Lepidapedidae	Bray *et al.* (1999)	AJ405261
Lepidapedon sommervillae Bray et Gibson, 1995	Lepidapedidae	Bray *et al.* (1999)	AJ405268
Lepidapedon zubchenkoi Campbell et Bray, 1993	Lepidapedidae	Bray *et al.* (1999)	AJ405269
Muraenolepitrema magnatestis Gaevskaya et Rodjuk, 1988	Lepidapedidae	This study	KY497958
Myzoxenus insolens (Crowcroft, 1945)	Lepidapedidae	Bray *et al.* (2009)	FJ788486
Neolepidapedon smithi Bray et Gibson, 1989	Lepidapedidae	Bray *et al.* (1999)	AJ405270
Postlepidapedon opisthobifurcatum (Zdzitowiecki, 1990)	Lepidapedidae	This study	KY497957
Postlepidapedon uberis Bray, Cribb et Barker, 1997	Lepidapedidae	Bray *et al.* (2009)	FJ788492
Profundivermis intercalarius Bray et Gibson, 1991	Lepidapedidae	Bray *et al.* (1999)	AJ405271
Outgroup			
Koseiria xishaense Gu et Shen, 1983	Enenteridae	Olson *et al.* (2003)	AY222233
Petalocotyle adenometra Hall et Cribb, 2000	Gyliauchenidae	Bray *et al.* (2009)	FJ788504

Distal ends of the membrane's sheets are connected to the proximal edge of the cirrus-sac. The cirrus-sac is 0.237 – 0.331 mm long and 0.05 – 0.06 mm maximal wide, and it is composed of internal seminal vesicle, pars prostatica, ejaculatory duct and eversible cirrus. The cirrus-sac is claviform, with a long proximal part accommodating the proximal part of the internal seminal vesicle and numerous ducts of the outer prostatic cells. The internal seminal vesicle is long, 50 – 70 % the length of the cirrus-sac, tubular, rectilinear or slightly twisted, and thin-walled. The pars prostatica is vesicular. The proximal half of the pars prostatica and distal end of the inner seminal vesicle are surrounded by a field of inner prostatic cells. The cirrus is unarmed and almost cylindrical. The female terminal genitalia are represented by a thick-walled metraterm running over dorsal or dorso-lateral surface of the cirrus-sac, and surrounded by aciniform groups of glandular cells. The length of the metraterm is 0.13 – 0.15 mm, which represents 40 – 60 % of the cirrus-sac length. The male and female canals open into the small genital atrium.

Phylogenetic analysis

Bayesian inference analysis based on sequences containing 900 bp produced topologies in which *P. opisthobifurcatum* formed a strongly supported clade with *Myzoxenus insolens* (Crowcroft, 1945), within a polytomic clade also composed of *Intusatrium robustum* Durio et Manter, 1968 and *Postlepidapedon uberis* Bray, Cribb et Barker, 1997 (Fig. 2a). In turn, the above-mentioned polytomic clade is weakly supported as a sister group to a large clade of lepidapedids, consisting of *M. magnatestis* and two monophyletic clades: *Lepidapedon* spp. and *Neolepidapedon smithi* Bray et Gibson, 1989 + *Profundivermis intercalarius* Bray et Gibson, 1991. The trematode *Bulbocirrus aulostomi* Yamaguti, 1965 is basal taxa to all other Lepidapedidae.

Bayesian inference analysis based on sequences containing 1230 bp has revealed that *P. opisthobifurcatum* and *M. insolens* formed a clade that was sister to *P. uberis* with low support (Fig. 2b) and in turn this clade composed of three species was sister to *I. robustum* with high support. The species *M. magnatestis* and *B. aulostomi* form a strongly supported sister clade to the above-mentioned group of lepidapedids. *Postlepidapedon opisthobifurcatum* and *P. uberis* were no more closely related than they were to other lepidapedids.

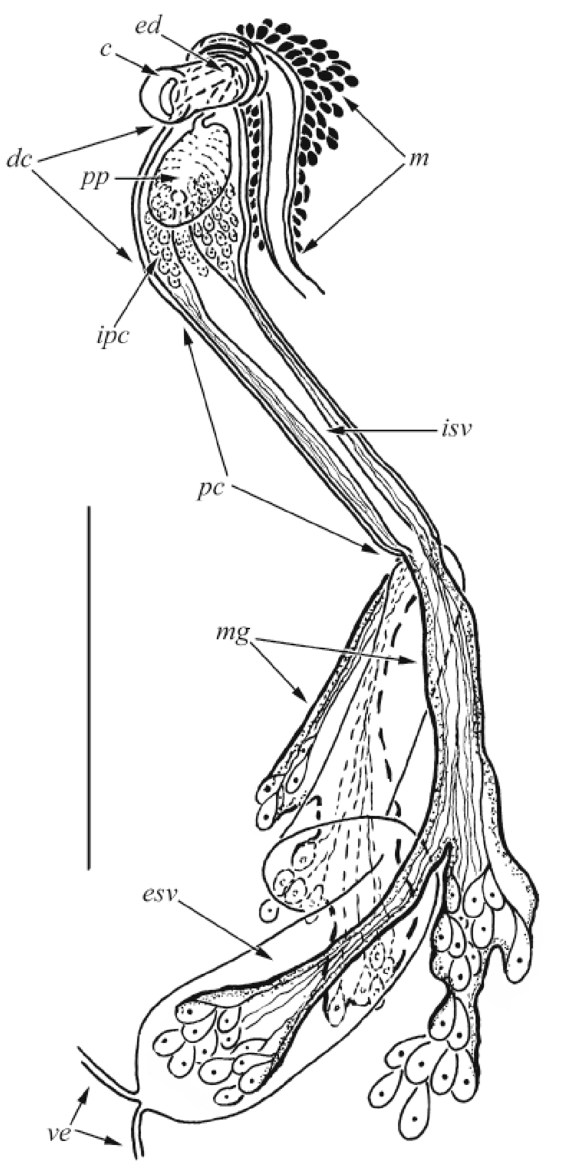

Fig. 1. Male and female terminal genitalia of *Postlepidapedon opisthobifurcatum* with cirrus everted through genital atrium, ventral view, *bar* 0.2 mm; *dc* distal part of cirrus-sac, *pc* proximal part of cirrus-sac, *c* cirrus, *ed* ejaculatory duct, *pp* pars prostatica, *ipc* inner prostatic cells, *isv* internal seminal vesicle, *mg* outer prostatic cells associated with thin-walled membrane, *esv* external seminal vesicle, *ve* vasa efferentia, *m* metraterm surrounded by glandular cells.

Discussion

According to Zdzitowiecki (1990, 1993), the cirrus-sac of *P. opisthobifurcatum* has an elongate-oval or clavate shape and it contains an elongate, but not exceptionally long, thin-walled internal seminal vesicle, a vesicular pars prostatica, a long and narrow ejaculatory duct, and a small cirrus. The external seminal vesicle is long and convoluted, and lies free in the parenchyma. The outer prostatic cells of *P. opisthobifurcatum* were not found by Zdzitowiecki (1990, 1993). However, the proximal edge of the cirrus-sac and the area in which the external seminal vesicle is located are difficult to observe in whole mounts of this parasite, because they are obscured by the ventral sucker, loops of uterus, and vitelline follicles. The study of the isolated terminal part of the reproductive system revealed outer prostatic cells associated with thin-walled open-ended membrane. The membrane, which covers some clusters of outer prostatic cells, is described for other lepidapedids, in particular *Paralepidapedon sebastisci* (Yamaguti, 1938), *M. magnatestis*, and also for some opecoelids (Shimazu & Shimura, 1984; Sokolov & Gordeev, 2015; Shimazu, 2016). Shimazu & Shimura (1984) consider it as the rudiment of the wall of the membranous sac (=proximal portion of cirrus-sac by Shimazu & Shimura's terminology), inherent for many lepidapedids and some opecoelids (see Bray, 2005; Cribb, 2005).

The position of *P. uberis* in the obtained phylograms (Fig. 2), separated from *P. opisthobifurcatum*, is consistent with differences in the morphology of the male terminal genitalia. *Postlepidapedon uberis* has a subglobular cirrus-sac with a small proximal part, containing a convoluted internal seminal vesicle. The ejaculatory duct is relatively short and thick-walled. The complex of the outer prostatic cells is absent in *P. uberis* (see Bray & Cribb, 2001). In addition, this species differs from *P. opisthobifurcatum* in the position of vitelline follicles. In *P. uberis* vitelline follicles form two lateral fields that are arranged in hindbody. These fields overlap the intestinal branches ventrally, laterally and dorsally (Bray & Cribb, 2001). In *P. opisthobifurcatum*, in addition to the lateral fields arranged in hindbody, there are two longitudinal intercaecal rows of the vitelline follicles that lie on the dorsal side of the body. Lateral fields of vitelline follicles in this species overlap the intestinal branches only ventrally and partly laterally (Zdzitowiecki, 1990).

Four other species of the genus *Postlepidapedon*: *Postlepidapedon philippinense* Machida, 2004, *Postlepidapedon secundum* (Durio et Manter, 1968), *Postlepidapedon spissum* Bray, Cribb et Barker, 1997, and *Postlepidapedon quintum* Bray et Cribb, 2001 also differ from *P. opisthobifurcatum* in the shape of the cirrus-sac (oval or elongate-oval without detached proximal part) (Bray *et al.*, 1997; Bray & Cribb, 2001; Machida, 2004). The species *P. philippinense, P. secundum,* and *P. quintum* do not have a complex of the outer prostatic cells. The distal end of the external seminal vesicle of *P. spissum* is encircled by glandular cells, lying unconfined in the parenchyma. (Bray *et al.*, 1997). Moreover, *P. secundum* has a coiled thin-walled internal seminal vesicle, while *P. spissum* and *P. quintum* have a rectilinear, but thick-walled, internal seminal vesicle. *Postlepidapedon philippinense* has rectilinear thin-walled internal seminal vesicle (Machida, 2004). The placement of lateral fields of the vitellarium relative to the intestinal branches in *P. quintum P. secundum,* and *P. spissum* is the same as in *P. uberis* (Bray *et al.*, 1997; Bray & Cribb, 2001). Accurate information about mutual location of lateral fields and intestinal branches in *P. philippinense* is absent (see Machida, 2004). We think it is likely that

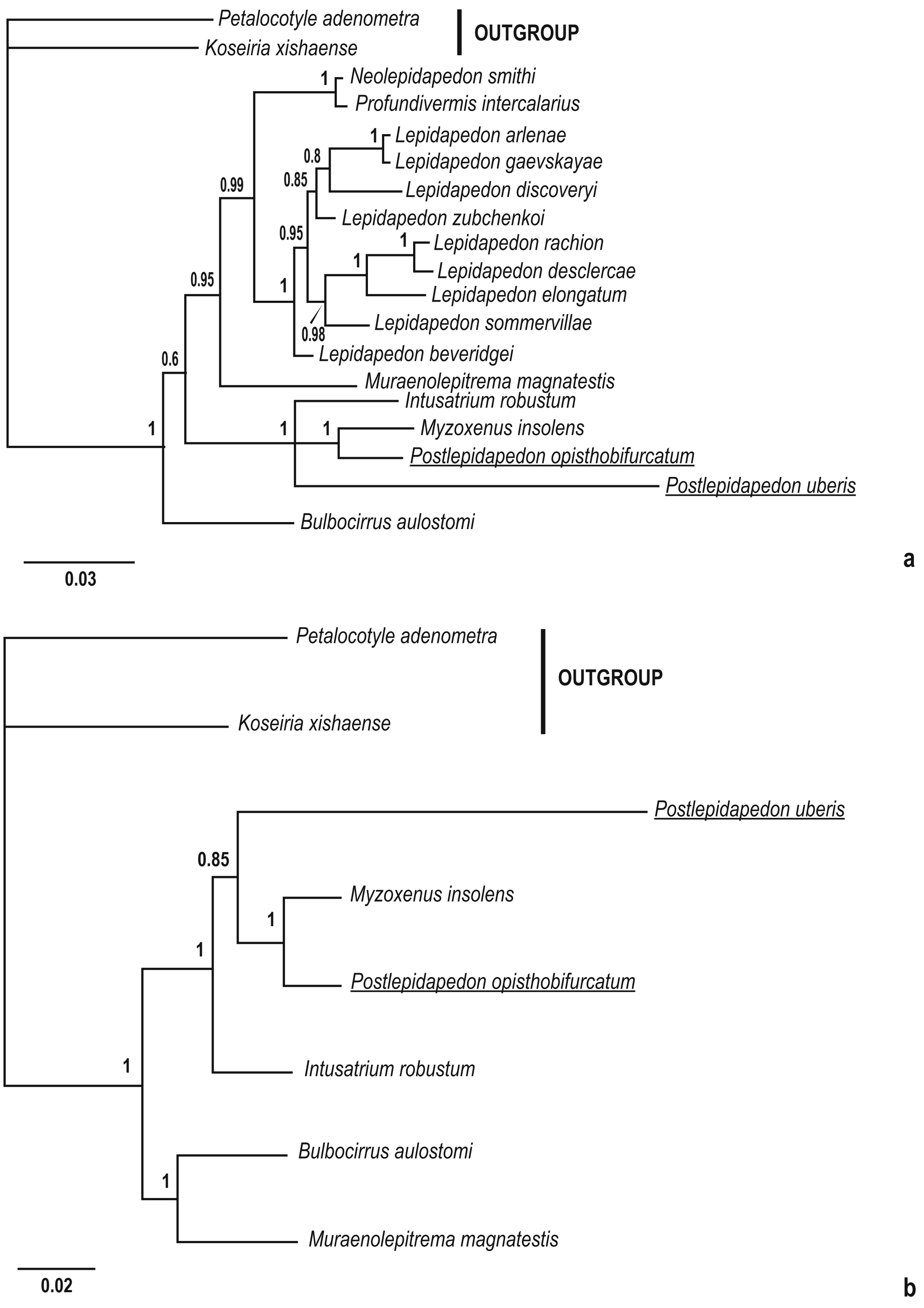

Fig. 2. Phylogenetic position of *Postlepidapedon opisthobifurcatum* within the Lepidapedidae based on 28S rDNA sequences containing 900 bp (a) and 1230 bp (b) analysed by Bayesian inference; nodal numbers indicate posterior probabilities. Scale bar shows substitutions per site.

only *P. opisthobifurcatum* among all the species currently recognised as representatives of genus *Postlepidapedon* will ultimately be proven to belong to this genus.

Phylogenetic analysis supports the position of *M. insolens* as the sister taxon of *P. opisthobifurcatum.* This result is unexpected because the indicated species are similar only by the signs common to many lepidapedids (see Bray & Cribb, 1998, 2012; Bray, 2005). Definitive hosts of *P. opisthobifurcatum* are gadiform fishes, and for *M. insolens* – perciform fishes (Bray & Cribb, 1998). Life cycles of these parasites are not known. In turn, *P. opisthobifurcatum* + *M. insolens* clade form a well-supported monophyletic group with the species *I. robustum* and *P. uberis*. In the phylogenetic model of Lepocreadioidea proposed by Bray *et al*. (2009) using partial 28S rDNA and *nad1* sequences, this species group (without *P. opisthobifurcatum*) was named "clade III." Bray *et al*. (2009) noted the absence of a general morphological synapomorphy in representatives of the clade III (at least for adult specimens); therefore, taxonomic reorganisation or revision of this clade is premature.

Conflict of Interest

Authors state no conflict of interest.

Acknowledgements

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The anti-parasitic effect of probiotic bacteria *via* limiting the fecundity of *Trichinella spiralis* female adults

B. BUCKOVÁ[1], Z. HURNÍKOVÁ[1], A. LAUKOVÁ[2], V. REVAJOVÁ[3], E. DVOROŽŇÁKOVÁ[1]*

[1]Institute of Parasitology, Slovak Academy of Sciences, Hlinkova 3, 04001 Košice, Slovakia, *E-mail: *dvoroz@saske.sk*;
[2]Institute of Animal Physiology – Centre of Biosciences, Slovak Academy of Sciences, Šoltésovej 4, 040 01 Košice, Slovakia;
[3]University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice, Slovakia

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Summary

A potential protective effect of probiotic strains against zoonotic *Trichinella spiralis* infection was investigated in the framework of a new therapeutic strategy aimed at using probiotics to control parasitic zoonoses. The study was focused on the impact of six selected probiotic (bacteriocinogenic) strains on the intensity of *T. spiralis* infection and female fecundity *ex vivo* and *in vitro*. Bacterial strains of different origin (*Enterococcus faecium* EF55, *Enterococcus faecium* 2019 = CCM7420, *Enterococcus faecium* AL41 = CCM8558, *Enterococcus durans* ED26E/7, *Lactobacillus fermentum* AD1 = CCM7421, *Lactobacillus plantarum* 17L/1) were administered daily in a dose of 10^9 CFU/ml in 100 µl, and mice were infected with 400 *T. spiralis* larvae on day 7 of treatment. Female adults of *T. spiralis* were isolated on day 5 post infection (p.i.) and subsequently were used in fecundity test *ex vivo*. *E. faecium* CCM8558, *E. faecium* CCM7420 and *E. durans* ED26E/7 strains significantly reduced the number of adults in the intestine. The application of *L. fermentum* CCM7421, *L. plantarum* 17L/1, *E. faecium* CCM8558 and *E. durans* ED26E/7 caused a significant decrease in the number of muscle larvae. The treatment with *E. faecium* CCM8558 and *E. durans* ED26E/7 showed the highest inhibitory effect on female fecundity (94 %). The number of newborn larvae (NBL) was also significantly decreased after administration of *L. fermentum* CCM7421 and *L. plantarum* 17L/1 (80 %). A direct impact of probiotic strains on female reproductive capacity was examined *in vitro* in females isolated from untreated infected mice on day 5 p.i. A correlation was found between the inhibitory effect and the concentration of probiotic strains. The reduction effects of the strains manifested as follows: *L. fermentum* CCM7421 (93 %), *E. faecium* CCM8558, *L. plantarum* 17L/1, *E. faecium* EF55 (about 80 %), *E. faecium* CCM7420 and *E. durans* ED26E/7 (about 60 %).
Keywords: *Trichinella spiralis*; female fecundity; probiotic bacteria; *Enterococcus*; *Lactobacillus*

Introduction

The host gut represents a complex ecosystem where the interactions between intestinal microbiota, immune system, and pathogens occur. For healthy organisms is crucial to form the balance between the gut microbiota and the host organism (Berrilli *et al.*, 2012). It has been recognized that the excretory/secretory molecules produced by helminths may lead to significant alterations in the composition of the gut microbiota (Walk *et al.*, 2010; Li *et al.*, 2012). Gut microbiota products and metabolites also significantly influence the survival and the physiology of many parasites and, consequently, the outcome of parasitic infections. This suggests that probiotic bacteria can successfully reduce the pathogenicity of many parasites, probably through multiple mechanisms (Berrilli *et al.*, 2012; Travers *et al.*,

* – corresponding author

2011). The main mechanisms of probiotic actions include: enhancement of the gut epithelial barrier, increase of adhesion to intestinal mucosa and simultaneous inhibition of pathogen adhesion, competitive elimination of pathogens, production of anti-microbial molecules, and modulation of the immune system (Goudarzi *et al*., 2014).

Several studies have investigated the ability of probiotics to influence the course of different parasitic infections (Travers *et al*., 2011). Positive effects of probiotic bacteria reducing the parasite burden and pathological changes in experimental trichinellosis due to the activation of local and systematic immune responses were previously described (Bautista-Garfias *et al.,* 1999, 2001; Martínez-Gómez *et al*., 2009, 2011; El Temsahy *et al*., 2015; Dvorožňáková *et al*., 2016), in ascariasis (Solano-Aguilar *et al*., 2004), and toxocarosis (Basualdo *et al*., 2007). Probiotic bacterial strains can positively affect protozoan parasitic infections such as cryptosporidiosis, giardiasis, coccidiosis (Gargala, 2008; Alak *et al*., 1999; Pérez *et al*., 2001; Shukla and Sidhu, 2011). Probiotic bacterial strains are also being tested in the host protection against blood parasites like *Babesia, Plasmodium,* and *Trypanosoma* (Bautista-Garfias *et al*., 2005; Galdeano and Perdigón, 2006; Martínez-Gómez *et al*., 2006; Eze *et al*., 2012).

Trichinellosis is a serious food-borne parasitic zoonosis caused by the nematode of the genus *Trichinella*, which is characterized by an extremely wide host range and worldwide distribution (Bruschi, 2012; Goździk *et al*., 2017). In general, therapeutic approaches against trichinellosis can be divided into two groups: classic and alternative. The classic treatment includes the application of anthelmintics, primarily albendazole and mebendazole (Gottstein *et al*., 2009); however, the efficacy of these benzimidazole derivatives is limited by the following factors: 1) weak activity against encapsulated larvae, 2) low water solubility, 3) increasing anthelmintic resistance, 4) contraindication in children and pregnancy (Yadav and Temjenmongla, 2012). Therefore, the anti-parasitic potential of probiotic bacteria (El Temsahy *et al*., 2015), natural proteins (Othman *et al*., 2016), and substances such as myrrh, thyme or artemisinin (Attia *et al*., 2015; Abou Rayia *et al*., 2017) is being increasingly utilized in recent years.

The present study was designed to study the anti-parasitic effects of six different probiotic strains of lactobacilli and enterococci on the parasite burden in the host and on the fecundity of *T. spiralis* females.

Materials and Methods

Probiotic strains

The effects of the following bacteria were tested: bacteriocin-producing strains with probiotic properties (*Enterococcus faecium* EF55, *Enterococcus faecium* 2019 = CCM7420, *Enterococcus faecium* AL41 = CCM8558, *Enterococcus durans* ED26E/7, and *Lactobacillus plantarum* 17L/1) and probiotic strain *Lactobacillus fermentum* AD1 = CCM7421. All used strains are original isolates which were not previously used for this purpose.

Enterococcus faecium EF55 was isolated from the chicken crop and characterized at the Institute of Animal Physiology SAS – IAP SAS, Košice, Slovakia. The strain produces a thermo-stable bacteriocin EF55 (Strompfová *et al*., 2010).

Enterococcus faecium 2019 = CCM7420 is a rabbit-derived strain with probiotic properties, which produces enterocin 2019 (Ent 2019) (Pogány Simonová *et al*., 2013). It was isolated and characterized at IAP SAS, Košice, Slovakia and deposited in the Czech Culture Collection of Microorganisms, Brno, Czech Republic – CCM7420.

Enterococcus faecium AL41 = CCM8558 (isolated and characterized at IAP SAS, Košice, Slovakia and deposited in the Czech Culture Collection of Microorganisms, Brno, Czech Republic – CCM8558) is an environment-derived strain. The strain produces an enterocin M with a wide antimicrobial inhibitory spectrum and possesses probiotic properties (Lauková *et al*., 2012; Mareková *et al*., 2007).

Enterococcus durans ED26E/7 was isolated from traditional ewes milk lump cheese at the Research Dairy Institute, Žilina – RDI, Žilina, Slovakia; but identified, characterized and prepared for experiment at IAP SAS, Košice, Slovakia (Lauková *et al*., 2015).

Lactobacillus plantarum 17L/1 was isolated from stored ewes cheese (RDI, Žilina, Slovakia) but identified, characterized and prepared for experiment at IAP SAS, Košice, Slovakia (Lauková *et al*., 2013).

Lactobacillus fermentum AD1 = CCM7421 was isolated and characterized at IAP SAS, Košice, Slovakia and deposited in the Czech Culture Collection of Microorganisms, Brno, Czech Republic – CCM7421. It is a canine-derived strain possessing probiotic properties (Strompfová *et al*., 2008).

All used strains were evaluated according to the EFSA rules (Piskoríková, 2010). For the experiment they were prepared as follows: they were cultivated in MRS broth (Merck, Eppelheim, Germany) at 37 °C for 24 h. Broth cultures were centrifuged (30 min at 10,000*g*) and the sediment was resuspended in Ringer solution (Merck, pH 7.0) to a concentration of 10^9 colony forming units per ml (CFU/ml). The purity of the strains was checked by the standard microbiological method (ISO-International Organization for Standardization) by spreading dilutions in Ringer solution (Merck, pH 7.0) onto the selective medium ME-*Enterococcus* agar (ISO-15214, Difco, Detroit, USA) and/or MRS agar (Merck, Eppelheim, Germany). The cultures for application were stored at 4 °C.

Parasite

The reference isolate of *Trichinella spiralis* (ISS 004) (obtained and assigned codes from the Trichinella Reference Centre in Rome), maintained by serial passages in ICR mice at the Institute of Parasitology SAS, was used for the infection. Larvae were released by artificial digestion (1 % pepsin, 1 % HCl for 4 h at 37 °C; both from Sigma-Aldrich, Germany) of tissue following the protocol of Kapel and Gamble (2000) and kept in saline solution until inoculation of experimental mice.

Experimental design

The experiment was performed on pathogen-free eight week old male BALB/c mice (VELAZ, Prague, Czech Republic; n = 110) weighting 18 – 20 g. Mice were kept under a 12-h light/dark regime at room temperature (22 – 24 °C) and 56 % humidity on a commercial diet and water.

Animals were divided randomly into 7 groups: Control (n = 15) – *T. spiralis* infection without the administration of bacterial strains; Group 1 (n = 15) – *Enterococcus faecium* EF55 + *T. spiralis*; Group 2 (n = 15) – *E. faecium* CCM7420 + *T. spiralis*; Group 3 (n = 15) – *E. faecium* CCM8558 + *T. spiralis*; Group 4 (n = 15) – *E. durans* ED26E/7 + *T. spiralis*; Group 5 (n = 15) – *Lactobacillus fermentum* CCM7421 + *T. spiralis;* Group 6 (n = 15) – *L. plantarum* 17L/1 + *T. spiralis*. Probiotic strains were administered *per os* daily at a dose of 10^9 CFU/ml in 100 μl and mice were infected *per os* with 400 *T. spiralis* larvae/mouse on day 7 of treatment. Samples of the small intestine and muscles were obtained on days: 5, 11, 18, 25 and 32 p.i. For *ex vivo* fecundity test, adult *T. spiralis* females were isolated from the small intestine of three mice from each group on day 5 p.i.

In vitro fecundity test included mice (n = 5) without probiotic treatment and infected *per os* with 400 *T. spiralis* larvae/mouse. Similarly, female adults of *T. spiralis* were obtained from the small intestine on day 5 p.i.

Intestinal worm burdens

The intestinal phase of infection was investigated on days 5, 11 and 18 p.i. The small intestine was cut into 5 – 10 cm long pieces, placed into a sieve and incubated in conical pilsner glasses in 37 °C NaCl (0.9 % saline) overnight. After incubation, gut pieces were discarded and the worms in the sediment were counted under stereomicroscope at 60 x magnification (Leica S8APO, Leica Microsystems, Germany).

Isolation of muscle larvae

The muscle phase of infection was examined on day 18, 25 and 32 p.i. Whole eviscerated carcasses were minced and artificially digested (1 % pepsin HCl for 4h at 37 °C; both from Sigma-Aldrich, Germany), according to Kapel and Gamble (2000). Samples were allowed to settle for 20 min before the supernatant was discarded and the sediment was poured through a 180 μm sieve into a conical glass and washed with tap water. The sediment was finally transferred to a gridded Petri dish and counted using a stereomicroscope at 40 x magnification (Leica S8APO, Leica Microsystems, Germany). Depending on the density of larvae either a sub-sample or the whole sample was counted.

Obtaining of female adults for fecundity tests

The adult *T. spiralis* females were obtained according to Cabaj (1990). The small intestine was washed with PBS medium (pH 7.2), split longitudinally, cut into 1 cm long pieces, placed into a sieve over 50 ml beakers containing RPMI 1640 medium (Sigma-Aldrich, Germany) with antibiotics (100U/ml penicillin; 100U/ml streptomycin) and incubated in a water bath at 37 °C for 2 h. After incubation, gut pieces were discarded and worms in the medium were centrifuged in centrifuge tubes (Falcon, France) at 67*g* for 5 min. The sediment was finally transferred to a Petri dish and the female worms were identified using a stereomicroscope at 40 x magnification (Leica S8APO, Leica Microsystems, Germany).

Ex vivo fecundity test

The females isolated from the gut of treated and infected mice (4 from each mouse) were rinsed with the incubation medium, transferred to separate wells of a 24-well tissue culture plate (Falcon, France) containing RPMI 1640 medium (Sigma-Aldrich, Germany) supplemented with 3 % foetal bovine serum plus antibiotics (100U/ml penicillin; 100U/ml streptomycin). The plates were sealed with plastic wrap and incubated for 20 h at 37 °C in 5 % CO_2. NBL were counted in each well using an inverted microscope at 60 x magnification (Leica DM IL LED, Leica Microsystems, Germany). Results were expressed as the average number of NBL *per* one female parasite.

In vitro fecundity test

T. spiralis female adults were obtained from the small intestine of untreated infected mice on day 5 p.i. (30 from each mouse) and incubated afterwards in RPMI 1640 medium (Sigma-Aldrich, Germany) enriched with 3 % foetal bovine serum and selected probiotic strain (*E. faecium* EF55; *E. faecium* CCM7420; *E. faecium* CCM8558; *E. durans* ED26E/7; *L. fermentum* CCM7421; *L. plantarum* 17L/1) at different concentration (10^7, 10^5, 10^3 and 10^1 CFU/ml) or without strains (control) for 20 h at 37 °C in 5 % CO_2. NBL were counted in each well using an inverted microscope at 60 x magnification (Leica DM IL LED, Leica Microsystems, Germany). Results were expressed as the average number of NBL *per* one female parasite.

Statistical analysis

Statistical differences were assessed using one-way ANOVA, followed by *post hoc* Tukey's test (a value of $P<0.05$ was considered significant), which allowed comparison between each two groups at each time point. The analyses were performed using the Statistica 6.0 (Stat Soft, Tulsa, USA) statistical package.

Ethical Approval and/or Informed Consent

The research related to animals has been complied with all the relevant national regulations and institutional policies for the care and use of animals. The experimental protocol was in compliance with current Slovak ethical rules for animal handling and it was approved by the Animal Care Committee of the Institute of Parasitology SAS and the State Veterinary and Food Administration of the Slovak Republic (Ro-3184/14-221).

Table 1. Numbers of adult worms isolated from the small intestine of mice with probiotic treatment and *T. spiralis* infection.

Day post infection	*T. spiralis* (Mean ± S.D.)	*E. faecium* EF55 + *T. spiralis* (Mean ± S.D.)	*E. faecium* CCM8558 + *T. spiralis* (Mean ± S.D.)	*E. faecium* CCM7420 + *T. spiralis* (Mean ± S.D.)	*E. durans* ED26E/7 + *T. spiralis* (Mean ± S.D.)	*L. fermentum* CCM7421 + *T. spiralis* (Mean ± S.D.)	*L. plantarum* 17L/1+ *T. spiralis* (Mean ± S.D.)
5	295 ± 24	293 ± 38	244 ± 25	235 ± 35	256 ± 7	209 ± 21	326 ± 48
11	229 ± 37	160 ± 39	*107 ± 25	*112 ± 14	*142 ± 29	210 ± 27	192 ± 8
18	2 ± 2	0 ± 0	0 ± 0	0 ± 0	1 ± 2	40 ± 12	1 ± 2

*P < 0.05 - statistically significant differences from *T. spiralis* infected group without treatment

Table 2. Numbers of muscle larvae isolated from mice with probiotic treatment and *T. spiralis* infection.

Day post infection	*T. spiralis* (Mean ± S.D.)	*E. faecium* EF55 + *T. spiralis* (Mean ± S.D.)	*E. faecium* CCM8558 + *T. spiralis* (Mean ± S.D.)	*E. faecium* CCM7420 + *T. spiralis* (Mean ± S.D.)	*E. durans* ED26E/7 + *T. spiralis* (Mean ± S.D.)	*L. fermentum* CCM7421 + *T. spiralis* (Mean ± S.D.)	*L. plantarum* 17L/1+ *T. spiralis* (Mean ± S.D.)
18	2 ± 4	40 ± 9	47 ± 2	24 ± 6	28 ± 16	4 ± 2	65 ± 6
25	50,080 ± 4,931	37,060 ± 4,150	**13,220 ± 1,842	27,970 ± 7,212	*23,250 ± 5,938	**18,380 ± 2,039	**15,540 ± 2,191
32	54,069 ± 8,020	42,580 ± 7,750	*23,810 ± 799	46,950 ± 3,818	**29,080 ± 2,204	*26,272 ± 2,566	32,070 ± 6,463

*P < 0.05; **P < 0.01 - statistically significant differences from *T. spiralis* infected group without treatment

Table 3. *In vitro* fecundity of *T. spiralis* females isolated from mice without treatment and subsequently cultivated with probiotic strains – Numbers of newborn larvae.

Concentration of probiotic strains (CFU/ml)	Control (Mean ± S.D.)	*E. faecium* EF55 (Mean ± S.D.)	*E. faecium* CCM8558 (Mean ± S.D.)	*E. faecium* CCM7420 (Mean ± S.D.)	*E. durans* ED26E/7 (Mean ± S.D.)	*L. fermentum* CCM7421 (Mean ± S.D.)	*L. plantarum* 17L/1 (Mean ± S.D.)
0	58 ± 8						
10^1		39 ± 13	46 ± 14	36 ± 16	43 ± 11	32 ± 14	38 ± 23
10^3		24 ± 15	**23 ± 7	37 ± 11	38 ± 21	*25 ± 13	37 ± 10
10^5		*34 ± 10	*29 ± 9	*32 ± 8	**26 ± 7	**16 ± 8	37 ± 13
10^7		**14 ± 9	**12 ± 7	**22 ± 10	**23 ± 9	***4 ± 3	**13 ± 5

*P < 0.05
**P < 0.01
***P < 0.001 - statistically significant differences from *T. spiralis* females incubated in medium without probiotic strains (control)

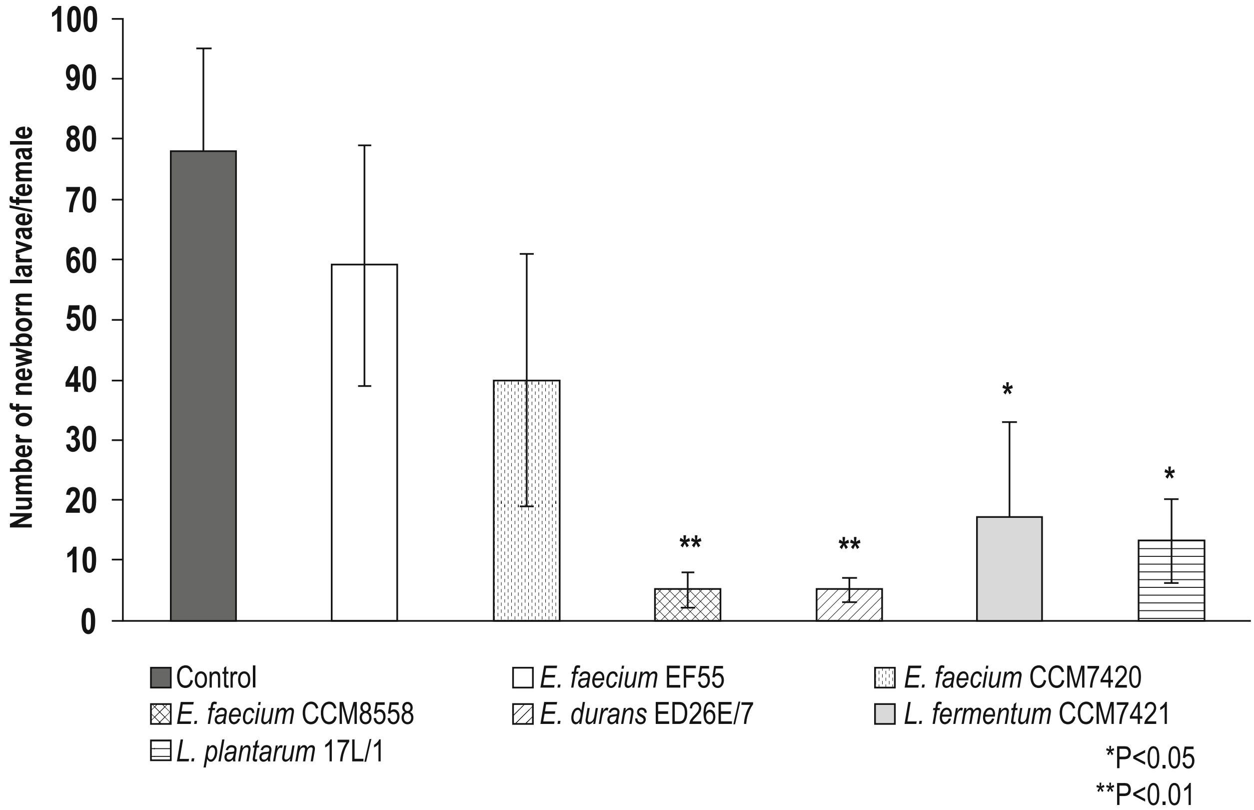

Fig. 1. *Ex vivo* fecundity of *T. spiralis* females isolated from mice treated with probiotic strains – Numbers of newborn larvae. *P<0.05; **P<0.01-statistically significant differences from *T. spiralis* infected group without probiotic treatment (control).

Results

Parasite burden – numbers of adults and muscle larvae

The highest numbers of *T. spiralis* adults (209 – 326) were found in the small intestine on day 5 p.i. in all groups (Table 1). A significant reduction of intestinal parasites occurred on day 11 p.i. in mice with administration of bacteriocin-producing strains *E. faecium* CCM8558 (107 ± 25), *E. faecium* CCM7420 (112 ± 14) and *E. durans* ED26E/7 (142 ± 29). Mice with this probiotic treatment absolutely eliminated adults from the small intestine till day 18 p.i. In evaluation of muscle phase of infection (Table 2), the occurrence of *T. spiralis* larvae was sporadic on day 18 p.i. (2 – 65). Numbers of muscle larvae reached the maximum in untreated mice on day 25 and 32 p.i. (50,080 ± 4,931 and 54,069 ± 8,020; respectively). Administration of strains *E. faecium* CCM8558, *E. durans* ED26E/7, *L. fermentum* CCM7421 and *L. plantarum* 17L/1 resulted in a significant larval count reduction with a higher efficacy on day 25 p.i. (13,220 – 23,250 larvae/mice).

Ex vivo fecundity test

In our experiments, female fecundity was significantly decreased after the administration of enterococci and lactobacilli in comparison to *T. spiralis* infected group without treatment (Fig. 1). The greatest inhibition in female reproductive capacity was caused by strains *E. faecium* CCM8558 and *E. durans* ED26E/7 with 94 % reduction of NBL. Similarly, the high reduction of female fecundity was recorded after treatment with *L. fermentum* CCM7421 and *L. plantarum* 17L/1 (78 % and 83 %). The application of strains *E. faecium* EF55 and *E. faecium* CCM7420 had only a modest inhibitory effect on the fecundity of females.

In vitro fecundity test

The strain concentration of 10^7 CFU/ml was the most effective among four examined concentrations of probiotic strains (Table 3). The highest decrease in the number of NBL was recorded after incubation of females with *L. fermentum* CCM7421 (93 %) followed by *E. faecium* CCM8558 (79 %), *L. plantarum* 17L/1 (78 %), *E. faecium* EF55 (76 %), *E. faecium* CCM7420 (62 %) and finally *E. durans* ED26E/7, which showed the 60 % reduction. The production of NBL was increased in relation to a decreasing concentration of bacteria. All tested probiotic strains at a concentration of 10^5 CFU/ml, except of *L. plantarum* 17L/1, had a significant inhibitory effect on female fecundity (in the range of 41 – 72 %). The females incubated with *E. faecium* CCM8558 or *L. fermentum* CCM7421 at a concentration of 10^3 CFU/ml were 60 % less fecund then control females. In comparison to the control, the lowest concentration of probiotic bacteria (10^1 CFU/ml) was also efficient to decrease the number of NBL.

Discussion

The available chemotherapy (benzimidazoles) of human trichinellosis is effective only against adult worms, not against muscle larvae. For trichinellosis as an important parasitic zoonosis with a worldwide distribution and epidemic occurrence (Devleesschauwer *et al.*, 2015), the development of new methods to control this disease is inevitable and the use of probiotic bacteria could be successfully employed (Martínez-Gómez *et al.*, 2011; El Temsahy *et al.*, 2015).

The nematode *T. spiralis* has been chosen as a model parasite to verify anti-parasitic properties of probiotic and bacteriocin-producing strains. Pathogenicity of *T. spiralis* is higher than other intestinal parasites due to the high production of NBL (Pozio *et al.*, 1992) and a strong immune response of the host (Pozio *et al.*, 1993; Bruschi *et al.*, 1999; Morales *et al.*, 2002). This study investigated the influence of tested probiotic bacteria on the adult worm and larvae burdens in mice.

T. spiralis infection affects the host in two phases, intestinal and muscular (Abou Rayia *et al.*, 2017). Parasite adults live in the epithelium of the small intestine, where the viviparous females produce a large number of NBL (500 – 1,500 larvae/female) from day 5 p.i. (Mitreva and Jasmer, 2006). These NBL migrate into the blood stream *via* intestinal lymphatics or mesenteric vessels, and finally reach the striated muscles that represent their predilection sites. There, they induce the formation of nurse cell and become encysted (Despommier, 1983). Gut microflora plays a crucial role in completing the life cycle of the parasite in the intestine, enabling the development into adults and their reproduction, and also in modulating the host immune response. Probiotic bacteria can provide an indirect protection, probably by modulating effect on newborn and muscle *T. spiralis* larvae (Travers *et al.*, 2011; Dvorožňáková *et al.* 2016).

Probiotic organisms are able to modulate their physicochemical environment: nutrients, pH, availability of receptors on epithelial cells, the epithelial tight junctions, and peristalsis. Probiotic bacteria can also control their biotic environment by regulating intestinal motility and mucus secretion (Gupta and Garg, 2009; Travers *et al.*, 2011), two major components of the intestinal physiology participating in the host defence against worms (Khan, 2008). The attachment of probiotics to the gut epithelium is an important determinant to achieve their beneficial effect on the host organism. All administered strains from our study sufficiently colonize the small intestine during the infection (Dvorožňáková *et al.*, 2014). In the present study, three strains of enterococci, *E. faecium* CCM8558, *E. faecium* CCM7420 and *E. durans* ED26E/7, significantly reduced the number of adult parasites in the intestine on day 11 p.i., with reduction rates of 53 %, 51 %, and 38 %, respectively. On the other hand, *L. fermentum* CCM7421 and *L. plantarum* 17L/1 had no influence on worm burden during the intestinal phase of the infection. We assume that it could be caused by the worse adhesive capacity of lactobacilli compared with enterococci (Lauková *et al.*, 2004). A weak anti-adult effect of *Lactobacillus* strains documented in our experiment is opposite to other studies. After intraperitoneal application of *L. casei* ATCC7469, Bautista-Garfias *et al.* (1999) recorded 88.5 % reduction in the number of *T. spiralis* adults. Also, when the same *L. casei* strain was administered *per os*, the reduction effect was 58 % (Bautista-Garfias *et al.*, 2001). Similarly, intraperitoneally applied strain of *L. casei* Shirota implied a 78.6 % reduction of intestinal parasites (Martínez-Gómez *et al.*, 2011). El Temsahy *et al.* (2015) recorded the reduction of *T. spiralis* adults after treatment with *L. plantarum* P16456 by 98 %, 65.4 % and 69 % on days 5, 12, and 17 p.i., respectively. These differences between the present study and other studies could result from using of various strains of lactobacilli, different infective and therapeutic doses, application method and/or design of experimental studies. We observed an increase in larval burden between days 25 and 32 p.i. in all experimental groups, untreated or treated mice. This might be caused by the continuous larval migration to the muscles. The increase was the lowest in untreated mice (4,000 larvae/mouse), and similar (5,000 - 10,000 larvae/mouse) in all four treated groups. Only in mice treated with *L. plantarum* 17L/1 and *E. faecium* CCM7420 - the numbers of muscle larvae increased by 17,000 -19,000 larvae/mouse, respectively. We can assume that probiotic therapy delayed migration of the NBL and larval motility was disrupted. Bacterial strains produce lactic and acetic acid, hydrogen peroxide, proteinaceous enterocins and bacteriocins, which are important mechanisms in pathogens exclusion (Šušković et al., 2010; Lauková et al., 2012). These bacterial substances might also affect the larvae vitality and participate in their destruction, particularly through hydrogen peroxide. It could be documented by the reduced larval burden. In our study, the number of muscle larvae in treated mice has significantly decreased on days 25 and 32 p.i., particularly in mice with administration of *L. fermentum* CCM7421, *L. plantarum* 17L/1, *E. faecium* CCM8558, and *E. durans* ED26E/7. The percentage of larval count reduction on day 25 was as follows: 63 %, 69 %, 74 %, and 54 %, respectively. Lower reduction values, yet still significant, were recorded on day 32 p.i.: 51 %, 41 %, 56 %, and 46 %, respectively. Similar efficacy against *T. spiralis* larvae was also shown in another probiotic strains, e.g. *L. casei* ATCC7469, *L. casei* Shirota, and *L. plantarum* P164 in a variety of experiments (Bautista-Garfias *et al.*, 2001; Martínez-Gómez *et al.*, 2011; El Temsahy *et al.*, 2015). In this context, it is important to emphasize that the beneficial effects of probiotics cannot be generalized given that they are strain-specific (Gupta and Garg, 2009).

The parasite infectivity is a result of the interplay of four components: the number of females that develop into adults, their fecundity, the length of their survival in the gut, and the period during which the muscle larvae remain viable (Dvorožňáková *et al.*, 2011). The decreased numbers of *T. spiralis* muscle larvae induced by bacterial strains in our study might be associated with a reduced female fecundity or destroying of NBL during their migration to the host muscles.

This is the first study that investigates the effect of probiotic strains on the fecundity of *T. spiralis* females. Our aim was to determine whether the reduced parasite burden is associated with a decreased fecundity induced by probiotic strains, or these strains prevented the NBL migration into the blood and the lymphatic circulation, or stimulated host immunity participated on this reduction. It could be discerned by results of *ex vivo* fecundity test at females isolated from the gut of infected and treated mice. The female reproductive capacity was significantly inhibited after administration of strains *E. faecium* CCM8558, *E. durans* ED26E/7 (about 94 %), *L. fermentum* CCM7421 and *L. plantarum* 17L/1 (about 80 %). In contrast to non-affected numbers of adults presented in the gut of mice treated with *Lactobacillus* strains, their reproductive capacity was suppressed. These strains did not affect the maturation of *T. spiralis* larvae into adults or their expulsion from the gut, but they contributed to the decreased muscle parasite loads in the host by the control of NBL production.

In vitro test regarding the fecundity of females *ex vivo* showed the extreme reduction in their reproductive potential. However, it may not reflect the fecundity *in vivo* where total muscle larval recovery lead to the lower reduction effects what was caused by probiotic therapy. Considering the numbers of larvae, which reached and encysted in muscles, the actual reproduction of females *in vivo* finished at about 50 %. These differences between *in vivo* and *ex vivo* female fecundity could be caused by biochemical and physiological conditions within the host organism. For example, the physico-chemical conditions of the jejunum are more fecund than those in the ileum. This site is more appropriate and results in a higher reproductive success of *T. spiralis* (Sukhdeo, 1991). Other authors (Gagliardo et al., 2002) confirmed that the intestinal life cycle of *T. spiralis* (including reproduction) is supported entirely by the host epithelial cells. All these supporting mechanisms provided by the host are absent under *in vitro* conditions. Based on our results, the impact of our six probiotic strains on female fecundity was elucidated; however, an influence of other factors within the host organism such as gut physiology or immunomodulatory activity of probiotic bacteria cannot be excluded. It could be related to the colonization of the intestinal epithelium with probiotic strains. The adhesion of strains on gut mucosal surfaces and also the production of antibacterial agents as bacteriocins, hydrogen peroxide (Pridmore *et al.*, 2008; Gupta and Garg, 2009; Hertzberger *et al.*, 2014) might prevent the parasite to enter the host epithelial cells, a site where *T. spiralis* larvae molt, ecdyse, develop to adulthood and reproduce (Gagliardo *et al.*, 2002).

Nevertheless, the data obtained from *in vitro* fecundity test also revealed a direct inhibitory impact of probiotic bacterial strains on female fecundity. The highest efficacy was detected after incubation of *T. spiralis* females with *L. fermentum* CCM7421 (93 % reduction of NBL) followed by strains *E. faecium* CCM8558, *L. plantarum* 17L/1, *E. faecium* EF55 (about 80 %), *E. faecium* CCM7420 and *E. durans* ED26E/7 (about 60 %). This may be related to the fact that the genera *Lactobacillus* and *Enterococcus* belong to the lactic acid bacteria, which in the process of glucose fermentation produce primarily lactic acid but also other organic acids, e.g. acetate and butyrate (Lauková *et al.*, 1998; Araújo and Ferreira, 2013; Azat *et al.*, 2016). These acids can decrease the local intestinal pH and thus directly disrupt the growth of the acid-sensitive organisms, including parasites (Mukhopadhyay and Ganguly, 2014). Results of the study of El Temsahy (2001) revealed that the acidic gastric pH led to a significant decrease in the fecundity of *T. spiralis* females both *in vivo* and *in vitro*. This was obvious by observing the inability of females to give birth to NBL and morphological changes of the reproductive organs, mainly the uterus, which could cause of the impairment in embryogenesis.

The resistance to *T. spiralis* infection is related to the ability of the host to prevent the development of infective larvae by removing adult worms from the small intestine, limiting the fecundity of adult females, and destroying NBL (Vasconi *et al.*, 2015). Our study confirmed the anti-parasitic effect of six selected probiotic strains using an accelerated kinetics of worm expulsion from the gut (*E. faecium* CCM8558, *E. faecium* CCM7420 and *E. durans* ED26E/7), the reduction in female's reproductive capacity (all examined strains), and by reduction of muscle larvae (*L. fermentum* CCM7421, *L. plantarum* 17L/1, *E. faecium* CCM8558 and *E. durans* ED26E/7). All these anti-parasitic mechanisms were strain-dependent and were not acting solely, but in cooperation with other host defence mechanisms. This idea has been confirmed by differences in obtained results in anti-parasitic parameters, where decreased presence of adult worms in the gut has not resulted in decreased numbers of muscle larvae and *vice versa*. The inhibited female fecundity played an important role in infected mice treated with *E. faecium* CCM8558 and *E. durans* ED26E/7. However, *in vitro* conditions revealed a strong effect against NBL production in strains *L. fermentum* CCM7421, *L. plantarum* 17L/1, *E. faecium* CCM8558, and *E. faecium* EF55. This effect was suppressed in the host environment by interactions between bacterial strains, host immune response, and inflammatory processes. Immune mechanisms involved in killing of the NBL include oxidative processes, eosinophil major basic protein or complement activation (Wang, 1997). Mast cells, eosinophils, neutrophils and macrophages are all able to adhere to the larvae surface and destroy NBL during *in vitro* incubation (Mackenzie *et al.*, 1981). Probiotic strains tested in this study modulated the immune response and stimulated phagocytosis and oxidative burst of blood leukocytes thus participating in the killing of larvae (Dvorožňáková et al., 2016).

Therapeutic approaches with the use of probiotic strains could help to reduce the risks of trichinellosis or complement classical anti-parasite treatments. Our study demonstrates that probiotic bacteria can provide strain-specific protection against *T. spiralis* nematode throughout reduced female fecundity. Several additional mechanisms involved in the anti-parasite defence should be further studied and elucidated to justify the therapeutic use of probiotics.

Conflict of Interest

The authors declare there is no conflict of interest relating to the information presented in this manuscript.

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In vivo nematicidal potential of camel milk on *Heligmosomoides polygyrus* gastro-intestinal nematode of rodents

D. ALIMI[1,2]*, A. ABIDI[1], E. SEBAI[1], M. REKIK[3], R. M. MAIZELS[4], M. DHIBI[1], H. AKKARI[1]

[1]Laboratoire de Parasitologie, Université de la Manouba, École Nationale de Médecine Vétérinaire de Sidi thabet, 2020, Tunisia, *E-mail: *dhouha.enmv@gmail.com*; [2]Faculté des sciences de Bizerte, Université de Carthage, 7021, Zarzouna, Tunisia; [3]International Center for Agricultural Research in the Dry Areas (ICARDA), P.O. Box, 950764 Amman 11195, Jordan; [4]Wellcome Trust Centre for Molecular Parasitology, Institute of Infection, Immunology and Inflammation, University of Glasgow, Sir Graeme Davies Building, 120 University Place, Glasgow G12 8TA, UK

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Summary

Following our previous findings on the *in vitro* anthelmintic effect of camel milk on *Haemonchus contortus*, the current study aimed at investigating its *in vivo* effect. Investigations were carried out using mice infected with *Heligmosomoides polygyrus* which is a parasite commonly used to test the efficacy of anthelmintics. Thirty six Swiss white mice of both sexes aged 5 – 6 weeks old, and weighing between 20 and 25 g were orally infected with 0.5 ml dose of 100, 1-week-old *H. polygyrus* infective larvae (L_3). After the pre-patent period, infected animals were randomly divided into 6 groups of 6 animals each. The nematicidal efficacy of camel milk was monitored through faecal egg count reduction (FECR) and total worm count reduction (TWCR). Four doses (8.25; 16.5; 33.0; 66.0 ml/kg body weight (bw)) for fresh camel milk and 22 mg/kg bw for albendazole were studied using a bioassay. Albendazole and 4 % dimethylsulfoxide were included in the protocol as reference drug and placebo, respectively. For all tested doses except 8.25 ml/kg bw, camel milk was effective *in vivo* against *H. polygyrus* reducing both faecal egg count and worm count ($p < 0.05$). The dose 66 ml/kg bw showed the highest nematicidal activity causing a 76.75 % FECR and a 69.62 % TWCR 7 day after initiating the treatment. These results support the possible use of camel milk in the control of gastro-intestinal helminthiasis.
Keywords: Camel milk; Faecal egg count reduction; *Heligmosomoides polygyrus*; Total worm count reduction

Introduction

The impact of gastrointestinal nematode (GIN) infection in small ruminants is linked to clinical signs associated with infection and also to subclinical economic losses (Martinez-Valladares *et al.*, 2015). Compared to other nematodes, *Haemonchus contortus* is one of the most abundant and prevalent gastrointestinal parasites in sheep and goats in Tunisia (Akkari *et al.*, 2013; Rouatbi *et al.*, 2016). The parasite can cause acute disease and high mortality in all categories of livestock. To date, the current mode of control of gastrointestinal parasitism relies on the repeated use of synthetic anthelmintics in combination with grazing management. However, the frequent use of these anthelmintics over many years leads to the emergence of drug resistant strains of parasites (Miller *et al.*, 2012). Even with optimally timed strategic treatments, this type of control is expensive, requires efficient health delivery systems particularly in remote production areas and, in most cases, is only partially effective (Ademola *et al.*, 2004). Therefore, there is an obvious need for, and significant global interest in the development of alternative improved means of controlling parasitic nematodes

* – corresponding author

(Britton *et al.*, 2015). In this respect, identifying therapeutically effective agents from natural products may reduce the risk of toxicity when the drug is used clinically and provide livestock farmers with environmentally friendly, easily accessible and not costly options (Lahlou, 2013).

Milk has shown positive results in controlling gastro-intestinal nematodes. Early reports suggested that milk exerted an anthelmintic effect on existing strongyle infections in foals (Leese, 1943) and on nematode infections in pigs (Spindler & Zimmerman, 1944; Spindler *et al.*, 1944; Shorb & Spindler, 1947). Calves fed entirely on milk had fewer, smaller *H. contortus* infection than calves fed “a normal diet” composed of cow’s milk, alfalfa hay and grain (Porter, 1941). In addition, lower numbers of *H. placei, Cooperia* spp. and *Oesophagostomum radiatum* were found in suckled calves than in weaned counterparts (Rohrbacher *et al.*, 1958). Milk proteins, or components associated with these proteins, reduced the motility of both sheathed and exsheathed L_3 *Teladorsagia circumcincta* in *in vitro* and *in vivo* studies performed by Zeng *et al.* (2001; 2003).

Camel milk is highly nutritious (Abbas *et al.*, 2013), and also has valuable medicinal and protective properties mainly due to its high concentration of immunoglobulins. Nutritional benefits of camel milk have been reported by several studies which included antihypertensive, hypoglycaemic (Agrawal *et al.*, 2003; 2011) and hypocholesterolaemic effects (Agrawal *et al.*, 2005). Moreover, camel milk is considered as an alternative to bovine milk for children who are allergic to bovine milk (Abusheliabi *et al.*, 2016; Al Haj & Al Kanhal, 2010; El-Agamy et *al.*, 2009). Scientific evidence is also building up that camel milk is unique for its therapeutic properties in terms of antioxidative factors (Kula, 2015), antibacterial (Benkarroum *et al.*, 2004), antiviral and antifungal activities (Yassin *et al.*, 2015; Abdel Galil & Alhaider, 2016).

In a preliminary study, our group demonstrated for the first time the *in vitro* nematicidal effect of camel milk against *H. contortus*, a gastrointestinal nematode of ruminants (Alimi *et al.*, 2016) and we were not aware of any published work investigating the *in vivo* anthelmintic effects of camel milk.

Therefore, the current study aimed to assess the *in vivo* nematicidal potential of camel milk against the rodent nematode, *Heligmosomoides polygyrus. H. polygyrus* belongs to the superfamily *Trichostrongiloidea* as do most nematodes of veterinary importance (Githiori *et al.*, 2003a; 2003b) and its biological cycle is easily maintained in the laboratory mouse (*Mus musculus*). *H. polygyrus* is a standard experimental model used for routine screening of potential drug candidates (Githiori *et al.*, 2003a).

Materials and Methods

Experimental Animals

Mice

Albino Swiss mice (*n* = 36), of both sexes age 5 to 6 weeks and weighing between 20 to 25 g, were used. Animals were obtained from the animal house of the Higher Institute of Biotechnology of Beja (University of Jendouba, Tunisia). Mice were housed in polypropylene cages with steel wire tops in an air conditioned room (22 ± 1 °C, 45 – 75 % relative humidity) maintained in a controlled atmosphere of 12 h light/12 h dark cycle. Food and water were provided *ad libitum*.

Helminth parasite

Infective third stage larvae (L_3) of *H. polygyrus* were generously provided by Dr. Rick Maizels, University of Edinburgh, UK. The parasite was cultured from the egg to L_3 stage in Petri dishes containing wet filter paper. Briefly, egg-containing faecal materials were macerated in the wet filter paper and incubated till they hatch into the first larval (L_1) stage, which underwent several stages of moulting before emerging as the third stage infective larvae (Adiel *et al.*, 2013).

Table 1. Faecal egg count (FEC) and % reduction of FEC at days 3, 5 and 7 after treatment with 4 % dimethylsulfoxide (DMSO), albendazole and different doses of camel milk.

Group	Dose	D_0	D_3	D_5	D_7
DMSO (4%)	-	11000 ± 9899	19000 ± 4242 (0.0)	16590 ± 1254 (0.0)	23870 ± 2440 (0.0)
Albendazole (mg/Kg bw)	22	26500 ± 2687	6161 ± 1328[c] (**67.57**)	13275 ± 4631 (**19.98**)	2940 ± 226[c] (**87.68**)
Camel milk (mL/Kg bw)	8.25	6403 ± 855	15111 ± 5727[a] **(18.42)**	13360 ± 1966 **(19.83)**	19420 ± 3012[a,b] **(18.73)**
	16.5	8705 ± 714	11187 ± 2022 **(42.11)**	12325 ± 318 **(25.32)**	15440 ± 636[a,b] **(34.23)**
	33	14300 ± 990	13203 ± 424 **(30.79)**	11905 ± 1124 **(28.24)**	10180 ± 318[a,b] **(56.57)**
	66	16910 ± 523	8022 ± 566[b] **(57.78)**	5350 ± 424[b] **(67.75)**	5550 ± 537[a,b] **(76.77)**

[a] p<0.05 comparison with positive control group (Albendazole)
[b] p<0.05 comparison with negative control group (DMSO 4 %)
[c] p<0.05 positive control group *vs*. negative control group

Test compounds
Preparation of the Albendazole solution
For the reference drug, albendazole (99.8 % pure standard reference, Médivét, S.A., Tunisia), 50 milligrams were diluted with 0.8 ml of DMSO and then distilled water was added to obtain the final volume of 50 ml. The obtained solution had a concentration of 1 mg/ml. A unique dose of 22 mg/kg bw of albendazole was administered. The 4 % DMSO was used in the *in vivo* assay as placebo (negative control) (Yondo *et al.*, 2013).

Camel milk
For 6 consecutive days in November 2016, camel milk samples were collected early morning from a camel farm located in the district of Sidi Bouzid (central Tunisia). Milk was recovered by hand milking. Samples were collected in sterile screw bottles and kept in cooling boxes (4 °C) until transported to the laboratory for immediate use.

Experimental design
Animals were screened for helminth parasites and subsequently treated with 7.5 mg/kg bw of albendazole to eliminate any roundworm infection. Then, the mice were randomly allocated into cages and allowed to acclimatize for 1 week. They had access to food and water *ad libitum.* In all studies, a dose of ≈100, 1-week-old *H. polygurus* infective larvae (L_3) was used to infect the mice, contained in 0.5 ml of distilled water. The mice were infected orally using an oral gavage needle (0.6 × 0.9 mm).
After the pre-patent period (9 to 11 days) (Smyth, 1996), infected mice were randomly allocated into 6 groups of 6 individuals each and treated as follows:
Group **1**: 4 % DMSO (negative control);
Group **2**: 22 mg of Albendazole kg^{-1} bw (positive control);
Group **3**: 8.25 ml of camel milk kg^{-1} bw;
Group **4**: 16.5 ml of camel milk kg^{-1} bw;
Group **5**: 33 ml of camel milk kg^{-1} bw;
Group **6**: 66 ml of camel milk kg^{-1} bw;
Groups 3 to 6 were treated orally with fresh camel milk at the different studied doses for 6 consecutive days (Day 9 to Day 14), while group 2 was treated with a single dose of albendazole (22 mg/kg bw) as standard anthelmintic (positive control) on Day 9.

Mice faecal samples
From Day 9 to Day 16 mice were isolated in individual cages to collect faecal pellets. For each mouse, a sample of faecal material was collected early in the morning before administration of the treatment on Days 9, 12, 14 and also on Day 16. Faecal pellets were immediately collected with a teaspoon, and placed in labelled Petri dishes containing 0.5 – 1 ml distilled water to prevent faecal materials from drying out. Faecal egg count was calculated as eggs per gram (EPG) of the faecal material according to the McMaster technique (Thienpont *et al.*, 1979). In brief, 2 g of this specimen was weighed, homogenized in a porcelain mortar and suspended in 60 ml saturated salt solution (0.4 % NaCl) (Thienpont *et al.*, 1979). Aliquots were mixed thoroughly with a Pasteur pipette and an equal volume of the suspension was introduced quickly under each of the two McMaster chambers (Hawksley, England) and viewed under a light microscope (10 x magnification).
The EPG was calculated according to the equation: (number of eggs counted x total volume)/ (volume counted x weight of faecal material).
The faecal egg count reduction (FECR) was determined by the following formula (Coles *et al.*, 1992): FECR (%) = 100 (1- T/C); T: means of FEC in the treated groups; C: means of FEC in the control groups.

Worm recovery
On Day 16 (8 days after the start of the treatments), mice were humanely euthanized using chloroform, and the body cavity was opened to remove the small intestine. This organ was placed in labelled Petri dishes containing 20 – 30 ml of distilled water and opened longitudinally with small scissors. The intestine was passed through the arm of a small forceps and the exudate containing parasites was washed in water (Githiori, 2004). The percentage of total worm count reduction (TWCR) was calculated by the method described by Enriquez (1993): TWCR (%) = 100 × (Total worm count in control group - Total worm count in treated group)/ Total worm count in control group.

Statistical analysis
The statistical analysis was done using STATVIEW v.5.0.1 software (SAS Institute, Cary, NC). The comparisons of means for FEC and TWC were done using analysis of variance (ANOVA) followed by Fisher's PLSD and all data were reported as mean± standard deviation. Differences were considered to be statistically significant when the p-value was less than 0.05.

Ethical Approval and/or Informed Consent

Mice were housed and maintained in a pathogen-free environment at the Department of Comparative Medicine. All experiments were performed according to the protocol No (NIH publication 86-23 revised 1985) USA, approved by (National Ethic Committee of Tunis University) IACUC.

Results

Faecal egg count reduction (FECR)
At D_0 of the administration of the treatment (corresponding to Day 9 of the experiment), mean FEC varied from 6403 ± 855 to 26500 ± 2687 (Table 1). For the lowest two doses of camel milk (8.25 and 16.5 ml/kg bw) and for the negative control (4 % DMSO) group, the mean FEC increased throughout the treatment period. This increase was highly significant ($p < 0.05$) when compared with groups that received fresh camel milk and albendazole. Treat-

Table 2. Mean worm intensity and % reduction of TWC at day 7 after treatment with 4 % dimethylsulfoxide, albendazole and different doses of camel milk.

Group	Dose (mg/kg)	Mean worm intensity after treatment ± standard deviation	% reduction of total worm count (TWCR)
DMSO	-	79.33±12.5[a]	0
Albendazole (mg/kg bw)	22	19.25±4[b]	**75.95**
Camel Milk (mL/kg bw)	8.25	70 .5±7.8[a,c]	**11.39**
	16.5	61.8 ±10.7[c]	**22.78**
	33	37.8±4.26[d]	**53.16**
	66	24.33±4.1[b]	**69.62**

a, b, c, d, numbers with the same letter in the same column are not significantly different at $p < 0.05$.
TWC: total worm count; TWCR: total worm count reduction; DMSO: dimethylsulfoxide

ment with albendazole was associated with a significant reduction in FEC ($p < 0.05$) starting day 3 post treatments, but this reduction was not significant on day 5. In this assay, albendazole was more active in comparison to the tested camel milk, but this commercial anthelmintic failed to show complete effectiveness (87.68 %) in infected mice. The dose rate 66 ml/kg bw for camel milk showed a nematicidal activity of (76.75 %). FECR was dose dependent.

Effects of camel milk and albendazole on the parasitic intensity of the nematode/Total worm count reduction (TWCR)

Albendazole was the most effective, causing a reduction of 75.95 % in TWC, while camel milk produced 69.62 % reduction at 66 ml/kg bw (Table 2). Results from albendazole and the highest dose of camel milk were not different ($p > 0.05$). Reduction of the TWC by camel milk was dose dependent (Table 2); the lowest dose rate (8.25 ml/kg bw) was associated with a TWC not different from the negative control (4 % DMSO), i.e. 0 % of TWCR (Table 2).

Discussion

In a preliminary study, *in vitro* tests have been undertaken and camel milk showed a nematicidal effect against *H. contortus,* a gastro-intestinal nematode of sheep, reducing egg hatching and adult worm motility by 100 % at a concentration close to 100 mg/ml (Alimi *et al*., 2016). The current study was performed to validate the anthelmintic activity of camel milk *in vivo* using *H. polygyrus.*

Our study revealed that, fresh camel milk significantly reduced the FEC and the TWC of *H. polygyrus*. This activity was more visible at the dose 66ml/kg bw by day 7 post-treatment, and resulted in a 76.75 % reduction of FECR and 69.62 % reduction of TWCR. This activity was dose and time dependent. We thought that, camel milk affect both the reproduction system of the worm and the infra-population. Also, our findings clearly demonstrated a reduction of parasite burdens in mice receiving camel milk; the reduction being evident 3 days after the start of the treatment. In fact, this reduction in egg count is an indication of reduced fecundity.

The possible explanation for such a decrease may be attributed to high amounts of proteins and peptides such as lysozyme (LZ), lactoferrin (LF), lactoperoxidase (LP), short peptidoglycan recognition protein (PGRP) all present in camel milk (Zeng *et al.*, 2001; 2003).

Camel milk is gaining popularity because of scientific reports of its high nutritional qualities and therapeutic value (Abusheliabi *et al.*, 2016). As such, camel milk composition has been widely studied throughout the world (Abbas *et al.*, 2013; Abu-Lehia, 1989; Alimi *et al.*, 2016; Asres & Yusuf, 2014; Konuspayeva *et al.*, 2009; Yadav *et al.*, 2015). The findings of the present study confirm the therapeutic activity of fresh camel milk on *H. polygyrus*, a nematode parasite infecting mice.

There are unfortunately no similar results in the literature using camel milk with which our results can be compared. Nevertheless, studies in sheep (Zeng *et al.*, 2001), cattle (Rohrbacher *et al.*, 1958; Satrija *et al.*, 1991), rabbits (Rohrbacher *et al.*, 1958), horses (Leese, 1943), and pigs (Shorb & Spindler 1947) have all demonstrated lower worm burdens in young mammals fed milk than in those weaned to solid feed or grass. Nevertheless, none of the previous studies tested camel milk.

Arguments to support the involvement of various components in milk have been adduced in some previous work; such benefits could accrue through a direct effect of milk on the nematode or indirectly through enhancement of the host immune response or of host resilience to the pathological effects of infection (Zeng *et al.*, 2003).

Direct effects could operate through specific effects of milk components, for example, of oligosaccharides on the adhesion of pathogens to host mucosa (Hakkarainen *et al.,* 2005), or of milk proteins and components associated with milk proteins on motility of nematode larvae (Zeng *et al.* 2003). However, indirect effects could operate via the superior amount and quality of proteins supplied by milk, which are protected from degradation in the rumen by the esophageal groove reflex, promoting greater or more rapid development of host immunity or greater host resilience to the pathogenic effects of infection; such effects were tested and confirmed in earlier works (Bown *et al.* 1991; Sykes & Coop, 2001).

Another indirect effect which has been put forward regarding the resistance to parasitism of milk-fed animals is the high pH of milk which was suggested to protect against nematodes. Indeed, high pH of milk has been suggested as a possible contributing factor to low worm burdens in milk-fed calves (Rohrbacher *et al.*, 1958) and is involved in increasing gut motility, hence causing expulsion of nematodes from skim-milk-fed pigs (Spindler *et al.*, 1944).

With regards the more specific anthelmintic effect of camel milk, Agrawal *et al*. (2002; 2005) put forward the hypothesis that high content of lactoferrin in camel milk, acts as a prebiotic having a strong physiological activity in the gastrointestinal tract. It has also been suggested that lactoferrin possesses antiparasitic activity towards a broad spectrum of species, such as *Pneumocystis carinii, Toxoplasma gondii* and *Tritrichomonas vaginalis*. (Cirioni *et al.*, 2000; Omata *et al.*, 2001). The antiparasitic effect of lactoferrin is predominantly linked to iron sequestration and destabilization of the parasite membrane (Elbarbary *et al.*, 2014).

The anthelminthic effects of camel milk may also be attributed to its antioxidant activity (Al-Humaid *et al.*, 2010). Camel milk possesses high levels of vitamins (B_2, C, and E) and is rich in mineral content (sodium, potassium, copper, magnesium, and zinc) (Al-Humaid et al., 2010; Nagy et al., 2013). Camel milk concentration in vitamin C is 3 to 5-fold higher than in bovine milk (Haddadin *et al.*, 2008; Salwa & Lina, 2010) and beyond its nutritional role; vitamin C exerts a powerful antioxidant activity (Abdel Galil *et al.*, 2016). In addition, the high minerals content in camel milk (Nagy *et al*., 2013) may act as antioxidant, and thereby removes free radicals (Powell, 2000; Kumar *et al.*, 2015).

Conclusion

This study has demonstrated the *in vivo* anti-parasitic effect of camel milk using the intestinal parasite *H. polygyrus* and its monogastric host the mouse with an observed reduction of faecal egg count by over 76 %. Our findings are backed by previous results from our laboratory on the *in vitro* anthelmintic effects of camel milk on *H. contortus.* While the *in vivo* anthelmintic effects of camel milk needs to be proven using ruminant species, current results may have important implications for the control of gastrointestinal parasites. Additional work is suggested (i) to identify camel milk components responsible of reducing the parasite burden, (ii) to elucidate their mechanism of action and (iii) to test their efficacy against a broader spectrum of helminth classes like trematode, cestode and nematodes.

Conflict of Interest

All authors declare no conflict of interest.

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Prevalence, abundance and intensity of eggs and oocysts of gastrointestinal parasites in the opossum *Didelphis virginiana* Kerr, 1792 in Yucatan, Mexico

R. A. ARAGÓN-PECH[1], H. A. RUIZ-PIÑA[1], R. I. RODRÍGUEZ-VIVAS[2], A. D. CUXIM-KOYOC[1], E. REYES-NOVELO[1]

[1]Centro de Investigaciones Regionales "Dr. Hideyo Noguchi", Universidad Autónoma de Yucatán, Av. Itzaes No. 491 por 59 Col. Centro, Mérida, Yucatán, México, C.P. 97000, E-mail: *A11017944@alumnos.uady.mx*, **rpina@correo.uady.mx*, *alan.cuxim.575@gmail.com*, *enrique.reyes@correo.uady.mx*; [2]Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Yucatán, Carretera Mérida-Xmatkuil Km. 15.5, Tizapán, 97100 Mérida, Yucatán, México, E-mail: *rvivas@correo.uady.mx*

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Summary

Virginia opossum, *Didelphis virginiana*, is a synanthropic mammal associated with peridomestic areas of Yucatán, However, little is known about the gastrointestinal parasite infections of this species. The infection prevalence, mean abundance and mean intensity of eggs and oocysts of gastrointestinal parasites, in opossums captured in the peridomestic areas were estimated in six rural localities of Yucatán, Mexico. Eighty-four faecal samples were processed by flotation technique. McMaster test was used to estimate the number of helminth eggs and protozoa oocysts per gram of feces. Seven genera of gastrointestinal parasites were identified, and then infection prevalence was estimated as follows: Protozoa *Eimeria* sp. (51.9 %) and *Sarcocystis* sp. (1 %); nematodes *Ancylostoma* sp. (80.56 %), *Cruzia* sp. (62.04 %), *Trichuris* sp. (60.19 %), *Capillaria* sp. (29.63 %), *Turgida* sp. (23.15 %), *Toxocara* sp. (11.11 %), and *Ascaris* sp. (1.85 %); and one acanthocephalan: *Oligacanthorhynchus* sp. (14.81 %). This is the first study on the diversity of gastrointestinal parasites in Virginia opossums, and first evidence about the potential role of opossums in the transmission of zoonotic gastrointestinal parasites in peridomestic areas of Yucatán, Mexico.
Keywords: Gastrointestinal parasites; *Didelphis virginiana*; Yucatán; Mexico

Introduction

Virginia opossum, *Didelphis virginiana*, is widely distributed across North and Central America and it can be found across a broad range of habitats up from Nearctic in southern Canada to the Neotropics in Costa Rica with the exception of arid zones in Mexico and the United States (Gardner, 2005). Opossums are synanthropic species, able to occupy habitats with high levels of disturbance, and, for this reason they are frequently found in agricultural, urban, and rural areas (Ruiz-Piña & Cruz-Reyes, 2002; Krause & Krause, 2006; Ruiz-Piña, 2010). This characteristic is relevant due to the diversity of parasites and pathogens being capable to infect this species, and suggest a reservoir role for many of them (Acha & Szyfres, 1988).

Few studies have been carried out in Mexico on the occurrence of gastrointestinal parasites (GIP) in *D. virginiana*. Cañeda (1997) recorded 17 parasite species from 10 opossums collected in Veracruz State where the most commonly found species were *Cruzia tentaculata, Turgida turgida, Trichuris didelphis,* and *Oligacanthorhynchus tortuosa*. Monet *et al*. (2005) found 19 helminth taxa (5 digeneans, 1 cestode, 2 acanthocephalans, and 11 nematodes) in *D. virginiana* captured in 10 Mexican states, in which *T. turgida*, and *C. tentaculata* were the most abundant parasites. The most recent study in Mexico, carried out by Acosta-Virgen *et al*. (2015), identified adult parasites in 40 sacrificed opossums from 12 Mexican states. They recorded 21 helminth taxa (6 trematodes, 2 cestodes, 3 acanthocephalans and 10 nematodes), which increased the diversity of intestinal parasite species for *D. virginiana* up to 41.

* – corresponding author

Studies on intestinal parasites of *D. virginiana* from southeastern Mexico are scarce (Cañeda, 1997; Monet *et al*., 2005; Acosta-Virgen *et al*., 2015).

In order to improve the understanding of gastrointestinal parasites infecting of this marsupial species the objective of this study was to evaluate the frequency of GIP infections, as well as assess the mean abundance and mean intensity of eggs and oocysts of GIP identified in feces of synanthropic specimens of *D. virginiana* captured in peridomestic areas of rural dwellings of six localities of northern Yucatán, Mexico.

Material and Methods

Study area

The present study was carried out in six localities: Cacalchén (N20°58'56" W89°13'40"), Homún (N20°44'19" W89°17'06"), Komchén (N21°06'13" W89°39'45"), Motul (N21°05'42" W89°16'59"), Tetiz (N20°57'44" W89°56'02") and Kopomá (N20°38'52" W89°53'55") located in northern Yucatán, Mexico. The altitude ranged between 3 and 20 meters above the sea level. The geology of the region is calcareous (karst) with strong superficial and internal water dissolution. The climate is warm sub-humid, with rains in summer. The native vegetation of the zone is a transition of medium and low tropical deciduous forest, but currently most of the area is covered by secondary vegetation (Flores & Espejel, 1994; Orellana *et al*., 2010). Rural localities ranged between 2000 – 6500 inhabitants. Except for Motul which has 23000 inhabitants, but preserves its rural housing characteristics.

Capture of opossums

The capture of opossums was carried out on monthly basis between July 2015 and January 2016. In each locality, 100 households per month were selected, with authorization of landowners or residents, and the opossums were captured with livetraps (66 × 23 × 23 cm, Tomahawk Live Trap Co.) baited with pineapple and placed in the peridomestic area at each house at dusk. Animals were collected on the following morning. Overall this represented a total of 600 trap/nights of capture effort.

We recorded the sex, age and weight of captured individuals and collected a stool sample. *D. virginiana* were captured and handled under conditions that minimized the stress and employed animal welfare procedures (NOM-062-1999; Sikes, 2016).

Fecal samples

Opossums are mammals that are characterized for their thanatosis behavior, generally described by defecation or urination during handling (Krause & Krause, 2006). Fecal sampling was performed as described by Rodríguez and Cob (2005). Approximately two to three grams of feces were obtained from each individual. Fecal specimens were placed in ziploc bags (12.5 × 8 cm) and/or sterile bottles and then stored in a cooler on ice during transportation to the laboratory. They were kept in refrigeration at 4 °C until their coprological analysis performed within 24 h.

Coproparasitological analysis

The coproparasitological examination consisted of macro and microscopic observations of feces to detect the GIP. A qualitative diagnosis was carried out by the flotation enrichment technique with saturated glucose solution (SSG) described by Rodríguez and Cob (2005). A quantitative diagnosis was made by applying of the modified McMaster technique of Rodríguez and Cob (2005), i.e. adding 1 g of feces and 14 ml of SSG. Fecal samples positive for coccidia for sporulation and identification were incubated for 10 days at 24 °C with 2.5 % potassium dichromate (Duszynski & Wilber, 1997).

Table 1. Infection parameters recorded in 84 fecal samples of *Didelphis virginiana* from six rural localities in northern Yucatán, Mexico.

Parasite	Infected animals (Prevalence, C.I. 95 %)	Mean abundance of eggs per gram (C.I. 95 %)	Mean intensity (C.I. 95 %)
Protozoa			
Eimeria sp.*	38 (45.2 %, 34.3 – 56.5)	18800 (5530 – 66600)	41600 (12700 – 151000)
Nematoda			
Trichuris sp.	49 (58.3 %, 47.1 – 69)	180 (115 – 322)	309 (206 – 531)
Capillaria sp.	24 (28.6 %, 19.2 – 39.5)	38 (22 – 73.8)	133 (87.5 – 227)
Ancylostoma sp.	71 (84.5 %, 75 – 91.5)	563 (43 – 736)	666 (511 – 858)
Cruzia sp.	52 (61.9 %, 50.7 – 72.3)	207 (149 – 291)	334 (255 – 459)
Ascaris sp.	2 (2.4 %, 0.3 – 8.3)	5.36 (0 – 20.8)	225 (100 – 225)
Toxocara sp.	5 (6.0 %, 2.0 – 13.3)	19.6 (5.36 – 48)	330 (160-510)
Turgida sp.	12 (14.3 %, 7.6 – 23.6)	22 (10.7 – 40.5)	154 (100 – 212)
Acantocephala			
Oligacanthorhvnchus sp.	14 (16.7 %, 9.4- – 26.4)	237 (62.5 – 727)	1420 (397 – 3750)

C.I. = confidence intervals. *Mean abundance is in oocysts per gram

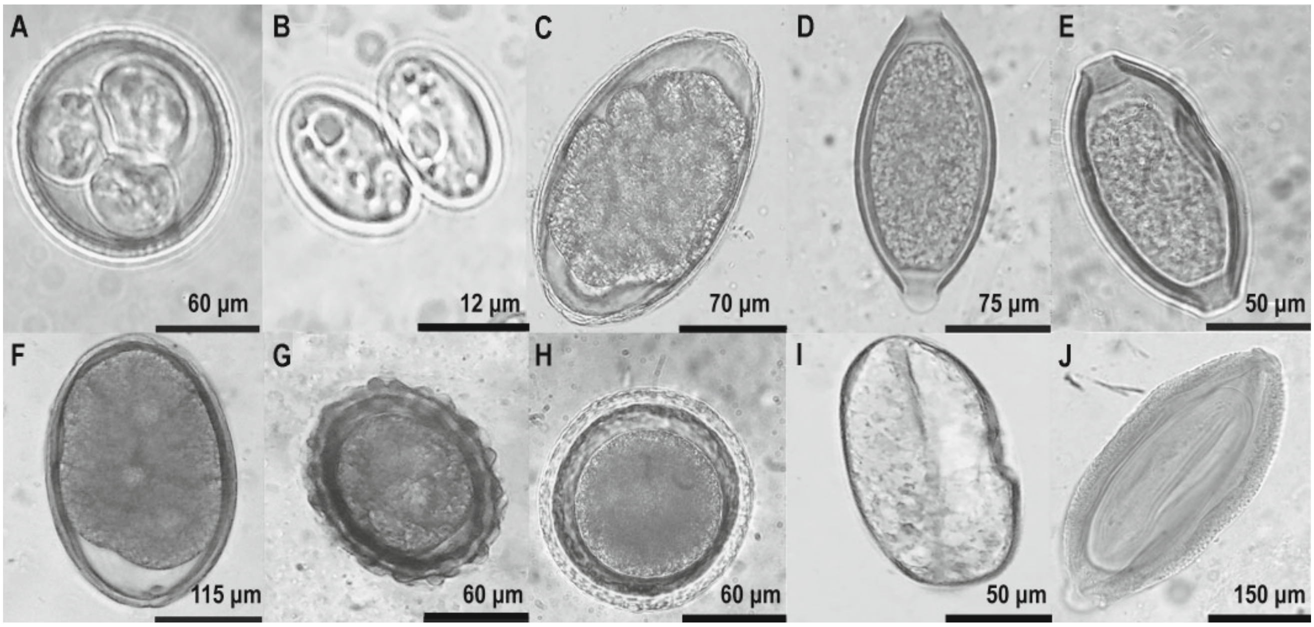

Fig. 1. Helminth eggs found in *Didelphis virginiana* from six rural localities in northern Yucatán, Mexico. **A** – Sporulated oocysts of *Eimeria* sp. (100x); **B** – oocyst of *Sarcocystis* s p. (100x); **C** – egg of *Ancylostoma* sp. (40x); **D** – egg of *Trichuris* sp. (40x); **E** – egg of *Capillaria* sp. (40x); **F** – egg of *Cruzia* sp. (40x); **G** – egg of *Ascaris* sp. (40x); **H** – egg of *Toxocara* sp. (40x); **I** – egg of *Turgida* sp. (40x); **J** – egg of *Oligacanthorhynchus* sp. (40x). The thick black line represents the scale used for each image.

Taxonomic determination

The morphological study of the eggs and oocysts of GIP was performed with light microscopy at 40× and 100× magnification (Zeiss [Axiostar]). Measurements were taken with the aid of a calibrated micrometer eyepiece. All measurements and scales of the images were measured in microns. The taxonomic determination was based on Garcia (2009), Zajac & Conboy (2012), and Bowman (2014). The identification of protozoan genera was based on the morphology of oocysts, their size, and the number of sporocysts (according to Rodríguez & Cob, 2005; Duszynski, 2016). The identification of the acanthocephalans was based on Petrochenko (1956).

Data analysis

For statistical analysis, the parameters prevalence (proportion of infected host with the traditional Clopper-Pearson CI), mean abundance (Bootstrap BCa), and mean intensity (Bootstrap BCa) were used. All summary statistics had 95 % confidence intervals, as proposed by Bush *et al*. (1997) and Rózsa *et al*. (2000), and were estimated with Quantitative Parasitology 3.0 (Reiczigel *et al*., 2013).

Ethical Approval and/or Informed Consent

This work does not involve human or experimentation with animals.

Results

A total of 84 *D. virginiana* were studied for counting the eggs/oocysts of GIP. The diversity of GIP found in *D. virginiana* was composed of two protozoa of the order *Eucoccidiida*, seven nematodes, and one acanthocephalan of the family *Oligacanthorhynchidae* (Table 1). The GIP eggs/oocysts were observed in 100 % of fecal samples (84/84). The oocysts of the protozoa *Eimeria* sp. (42.5 %) and *Sarcocystis* sp. (1.19 %) were found in 58.33 % (49/84) samples (Fig. 1A–B). Eggs belonging to seven genera of nematodes were found: *Ancylostoma* sp. (84.5 %, 71/84, Fig. 1C), *Trichuris* sp. (58.3 %, 49/84, Fig. 1D), *Capillaria* sp. (28.6 %, 24/84, Fig. 1E), *Cruzia* sp. (61.9 %, 52/84, Fig. 1F), *Ascaris* sp. (2.4 %, 2/84, Fig. 1G), *Toxocara* sp. (6 %, 5/84, Fig. 1H), *Turgida* sp. (14.3 %, 12/84, Fig. 1I). Finally, the eggs of the acanthocephalan *Oligacanthorhynchus* sp. (Fig. 1J) were found in 16.70 % (14/84) of positive samples.

Monthly variation in parasite prevalence showed the highest values for November and December, two months after the highest amount of rain registered in the region (Fig. 2).

The co-parasitism was recorded in the 91.6 % of the studied opossums (77/84). Table 2 shows the monthly variation in the prevalence of co-parasitism. Only 8.3 % (7/84) of the studied opossums were infected with a single parasite. However, a co-parasitism of 2 – 7 parasites were recorded (Table 2). The frequency of co-parasitism found in the studied opossums is presented in Table 3.

Discussion

The protozoan *Eimeria* sp. and the nematodes *Ancylostoma* sp., *Ascaris* sp. and *Toxocara* sp. represent new records for *D. virginiana* in Mexico (Cañeda, 1997; Monet *et al*., 2005; Acosta-Virgen *et al*., 2015).

Table 2. Monthly prevalence of co-infection of gastrointestinal parasites in *Didelphis virginiana* studied in peridomiciles from six rural localities in northern Yucatán, Mexico.

			2015			2016
Co-infection prevalence (n=77)	July	September	October	November	December	January
2 parasites	21.4	7.1	14.2	14.2	14.2	21.4
3 Parasites	21.7	4.3	13.1	34.7	13.1	13.1
4 Parasites	5.2	26.3	15.7	36.8	15.7	0
5 Parasites	0	20	10	20	50	0
6 Parasites	11.1	0	11.1	44.4	33.3	0
7 Parasites	0	0	25	25	50	0

Among the GIP found in this study, five nematodes with zoonotic potential were recorded: *Ancylostoma* sp., *Toxocara* sp., *Trichuris* sp., *Ascaris* sp., and *Capillaria* sp. The nematode *Ancylostoma* sp. represents the first report for *D. virginiana* in Mexico. Rueda *et al*. (2014) reported a frequency of 60 % (9/15) of *Ancylostoma* sp., in the feces of *D. marsupialis* in Colombia, with a lesser frequency that was found in the present study. In Brazil, Pinto *et al*. (2014) found *Toxocara cati* in the feces of *D. albiventris.*

The presence of *Ancylostoma* sp., and *Toxocara* sp. in the feces of *D. virginiana* could be due to the high abundance of these parasites found in other species of animals such as dogs and cats living in the peridomestic areas of rural households in the studied region (Rodríguez *et al*., 2001; Rodríguez *et al*., 2011; Ortega *et al*., 2015). However, to verify this hypothesis it is necessary to conduct specific studies on the cross-transmission of these parasites between *D. virginiana* and domestic and wild animals.

The nematodes *Trichuris* sp., *Cruzia* sp. and *Turgida* sp. have been previously reported in Yucatán, and are commonly recorded in *D. virginiana* from other Mexican regions (Monet *et al*., 2005; Acosta-Virgen *et al*., 2015).

Due to omnivorous nature of *D. virginiana*, the infection with *Turgida* sp. and *Oligacanthorhynchus* sp. may have been caused due to the ingestion of intermediary hosts. In the case of *Turgida* sp., cockroaches are known to act as intermediate hosts (Anderson, 2000). Also, the myriapods are recognized as intermediate hosts for genus *Oligacanthorhynchus* (Richardson, 2006). These could

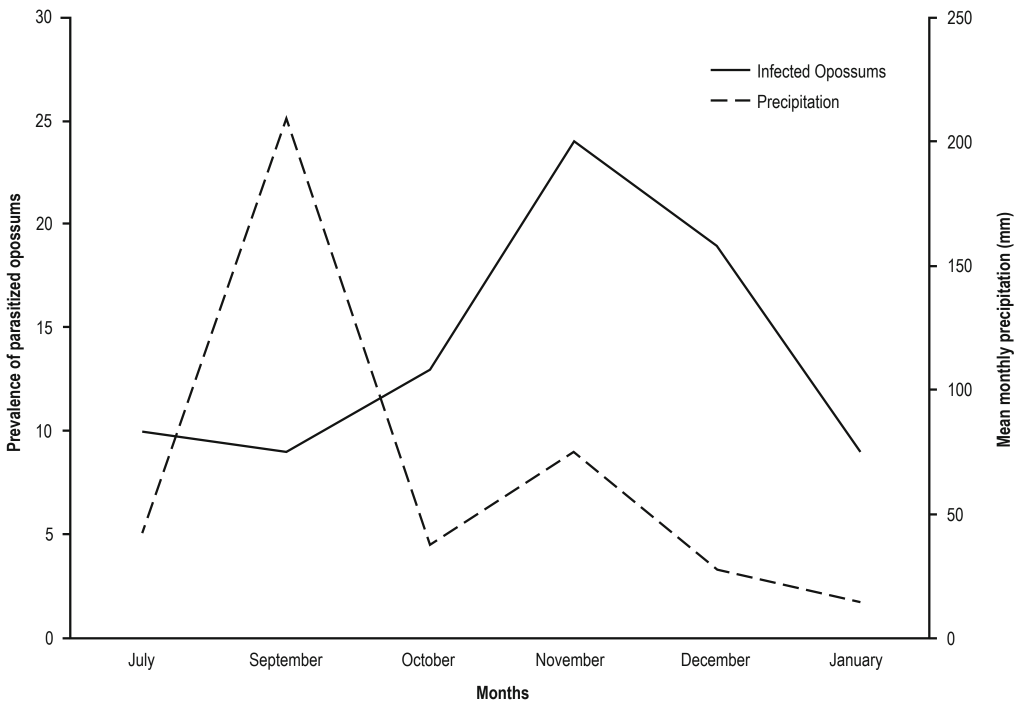

Fig. 2. Mean monthly precipitation registered from six rural localities in northern Yucatán, Mexico and the prevalence of gastrointestinal parasites in *Didelphis virginiana*

Table 3. Frequency of co-parasitism of gastrointestinal parasites for 77 opossum *Didelphis virginiana* from six rural localities in northern Yucatán, Mexico.

Nematoda	**Infected animals (%)**
Ancylostoma + *Cruzia*	8 (10.39)
Ancylostoma + *Trichuris*	3 (3.90)
Ancylostoma + *Capillaria*	1 (1.30)
Ancylostoma + *Turgida*	1 (1.30)
Capillaria + *Turgida*	1 (1.30)
Ancylostoma + *Cruzia* + *Trichuris*	8 (10.39)
Ancylostoma + *Capillaria* + *Trichuris*	2 (2.60)
Ancylostoma + *Cruzia* + *Turgida*	1 (1.30)
Ancylostoma + *Capillaria* + *Cruzia* + *Trichuris*	2 (2.60)
Ancylostoma + *Capillaria* + *Cruzia* + *Toxocara* + *Trichuris*	1 (1.30)
Protozoa + Nematoda	
Eimeria + *Ancylostoma*	2 (2.60)
Eimeria + *Trichuris*	2 (2.60)
Eimeria + *Ancylostoma* + *Trichuris*	5 (6.49)
Eimeria + *Ancylostoma* + *Cruzia*	3 (3.90)
Eimeria + *Trichuris* + *Turgida*	1 (1.30)
Eimeria + *Ancylostoma* + *Cruzia* + *Trichuris*	6 (7.79)
Eimeria + *Capillaria* + *Cruzia* + *Ancylostoma*	3 (3.90)
Eimeria + *Cruzia* + *Turgida* + *Ancylostoma*	2 (2.60)
Eimeria + *Ancylostoma* + *Capillaria* + *Cruzia* + *Trichuris*	2 (2.60)
Eimeria + *Ancylostoma* + *Capillaria* + *Cruzia* + *Turgida*	2 (2.60)
Eimeria + *Ancylostoma* + *Capillaria* + *Cruzia* + *Trichuris* + *Toxocara*	1 (1.30)
Eimeria + *Ancylostoma* + *Capillaria* + *Cruzia* + *Trichuris* + *Turgida*	2 (2.60)
Acantocephala +Nematoda + Protozoa	
Oligacanthorhynchus + *Trichuris*	1 (1.30)
Oligacanthorhynchus + *Capillaria*	1 (1.30)
Oligacanthorhynchus + *Cruzia*	1 (1.30)
Oligacanthorhynchus + *Cruzia* + *Ancylostoma*	2 (2.60)
Oligacanthorhynchus + *Turgida* + *Ancylostoma*	2 (2.60)
Oligacanthorhynchus + *Trichuris* + *Ancylostoma* + *Eimeria*	1 (1.30)
Oligacanthorhynchus + *Trichuris* + *Ancylostoma* + *Cruzia*	3 (3.90)
Oligacanthorhynchus + *Capillaria* + *Cruzia* + *Trichuris*	1 (1.30)
Oligacanthorhynchus + *Cruzia* + *Ancylostoma* + *Eimeria*	1 (1.30)
Oligacanthorhynchus + *Ancylostoma* + *Cruzia* + *Eimeria* + *Trichuris*	2 (2.60)
Oligacanthorhynchus + *Ancylostoma* + *Cruzia* + *Eimeria* + *Trichuris* + *Toxocara*	2 (2.60)
Oligacanthorhynchus + *Ancylostoma* + *Cruzia* + *Toxocara* + *Trichuris* + *Capillaria* + *Turgida*	1 (1.30)

be present in the studied localities, since there are at least 31 of myriapods species distributed in Yucatán (Bueno *et al*., 2004), and during the present study, some species were observed as frequent inhabitants of the peridomestic areas. Future research must consider also the collection and dissection of cockroaches and myriapods. thus confirm the presence of infectious stages of *Turgida* sp. and *Oligacanthorhynchus* sp., and explain the way how they contribute to the life cycle of GIP in *D. virginiana* from Yucatán.

The protozoan *Eimeria* sp. was one of the most frequent and abundant GIP in *D. virginiana*. This protozoan had not been previously reported in *D. virginiana* in Mexico. However, in Yucatán it was found having high frequency, detected in 45.2 % (38/84) of fecal samples, and was present in the six sampled localities. These results are discordant with those reported by Joseph (1974), who reported a lower percentage of infection (13 %, 2/15) in *D. virginiana* individuals infected with *Eimeria indianensis* in the state of Indiana, USA.

The parasites diversity found in *D. virginiana* could be explained as a result of the food source available in the peridomiciles. Due to opossums omnivorous and foraging behaviors, miscellaneous GIP eggs or larvae may be ingested or become exposed through the consumption of intermediate hosts (Krause & Krause, 2006; Ruiz-Piña *et al*., 2013). Another studies have shown that *D. virginiana*, consume feces of other animals (Gibson *et al*., 2003; Livingston *et al*., 2005). This can be important for the transmission of other parasites with direct life cycles. This is extremely relevant in view of the fact that opossums that inhabit the peridomiciles in Yucatán, interact with dogs, cats, pigs, cows, horses, among other mammals like chickens and other fowl. Domestic animals are susceptible to cross infection with GIP, what may also include many zoonotic diseases (Ruiz-Piña & Reyes-Novelo, 2012) like *Ancylostoma*.

In this context, opossums are frequent visitors and occupants of the peridomiciles in Yucatán, as a result of food and shelter availability in this ecotope (Ruiz-Piña *et al*., 2013). As a result they host the pathogens that circulate between those animals (Ruiz-Piña, 2010). This may represent a potential zoonotic risk to families that inhabit those localities. Primarily, because the use of the peridomicile environment for different activities such as washing clothes, keeping domestic animals, and also as a place for family reunions and recreational activities for children (Pacheco-Castro *et al*., 2013).

The higher prevalence of parasite eggs and co-parasitism in the months of November and December could be explained by parasite's life cycle. Taylor *et al*. (2016) explain that temperature (18 – 26 °C) and humidity (80 – 100 % relative humidity) are the most important factors involved in trichostrongyloids and strongyloids larval survival in the environment. These conditions are typical in Yucatán, during rainy season (June to October), (Orellana *et al*., 2010), and this could also explain that the higher GIP prevalence was recorded after the rainy season. Ruiz-Piña and Cruz-Reyes (2002) and Ruiz-Piña (2007) documented that weaned off juvenile opossums start roaming and looking for food in the peridomiciles at the beginning of the rainy season. So if they get infected with parasite larvae in that time of the year by November/December these population have adult parasites in their digestive tract and excrete eggs through fecal drops.

The present study is the first analysis on GIP diversity that interacts with *D. virginiana* in northern Yucatán, and the presence of this marsupial contributing to the dispersion of GIP with zoonotic potential in the peridomestic zones. In future studies, it would be necessary to apply molecular techniques for the taxonomic identification of GIP. Subsequently, the ecology of transmission and the role of *D. virginiana* in the life cycles of these GIP should be explored.

Conflict of Interest

Authors state no conflict of interest.

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Endoparasites of the European hare (*Lepus europaeus*) (Pallas, 1778) in central Italy

V. SERGI[1], G. ROMEO[2], M. SERAFINI[3], E. TORRETTA[3], F. MACCHIONI[1]*

[1]Department of Veterinary Science, University of Pisa, via Livornese lato Monte, 56122, Pisa, Italy, E-mail: **fabio.macchioni@unipi.it*, *sergi.valeria89@hotmail.it*; [2]Ufficio per le Attività Faunistico Venatorie e Ittiche dell'Amministrazione Regionale di Grosseto. Regione Toscana, Italia (Office for Hunting and Fishing Activities of Grosseto Regional Administration, Italy), E-mail: *giorgina.romeo@regione.toscana.it*; [3]Di.S.T.A., University of Pavia, Via Ferrata 1, 27100 Pavia, Italy, E-mail: *teos.teos@libero.it*, *torretta.elisa@gmail.com*

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Summary

Brown hare (*Lepus europaeus*) populations in Europe have declined through decades due to several, but not clear yet, factors. Parasite infections and diseases are some of the causes that directly affected the survival and breeding rates of animal population.
A study on the endoparasites of 70 hares (37 hunted free-living hares, and 33 bred on farms hares) was performed between 2015 - 2017 in the province of Grosseto (central Italy), an area where the impact of parasites in the hare population has never been investigated. During necroscopic analysis of hunted hares the following helminthes were found: *Trichostrongylus retortaeformis* (87.1 %), *Passalurus ambiguus* (12.9 %) and *Andrya* spp. (6.4 %) in the intestinal tract, *Protostrongylus cuniculorum* (8.3 %) in lungs and *Dicrocoelium dendriticum* (16.7 %) in livers. The prevalences of the intestinal helminthes in bred hares were: 12.1 % for *Passalurus ambiguus* and 3 % for *Trichostrongylus retortaeformis*. The coprological analysis showed prevalences of 64.9 % for coccidia in the 37 hunted hares and 45.5 % in the 33 bred hares. The relationship between the intensities of parasitic infections and body weight was evaluated.
The results of the present study in the Grosseto area indicate that free-living hares have few species of parasites and that the intensities of parasitic infection did not affect their general condition and health, suggesting that endoparasites played no detectable role in the dynamics of this hare population.
Keywords: Brown hare; necroscopical analysis; coprological analysis; host-parasite

Introduction

The European hare (*Lepus europaeus*) is the most important game species in Europe and the most widespread hare species in Italy. Since the 1960s, there has been a general decrease in the number of hares in many European countries (Smith *et al.,* 2005; Marboutin *et al.,* 2003). There are several probable causes of this population decline: a reduction in the number of suitable habitats for hares due to the intensification of agriculture (Smith *et al.,* 2005), over-hunting (Meriggi *et al.,* 2001), the increase in the number of predators, especially the red fox (*Vulpes vulpes*) (Goszczynski & Waseilewski, 1992; Knauer *et al.,* 2010), and the spread of infectious diseases such as EBHS (European brown hare syndrome), pasteurellosis, yersiniosis and tularemia (Lamarque *et al.,* 1996; Posauts *et al.,* 2015). Parasite infections can also be significant negative controlling factors of hare populations in terms of being the direct cause of death and, above all, as debilitating factors (Posauts *et al.,* 2015; Diakou *et al.,* 2014; Kornaś *et al.,* 2014; Chroust *et al.,* 2012; Dubinsky *et al.,* 2010).
The aim of the present study was to investigate the endopara-

* – corresponding author

sites of both free-living and bred European hares in the province of Grosseto (Tuscany, central Italy), an area where the hare population has already been well studied (e.g. Santilli *et al.,* 2014; Santilli & Ferretti 2008), with the exception of the impact of parasites, which has never been investigated. In Tuscany there has been a decline of the species since the late 1960s (Santilli & Galardi 2006). The last published data revealed a density of 9 hares/km^2 estimated in 23 protected areas in the province of Grosseto (Santilli &Ferretti, 2008).

Materials and Methods

A total of 70 European hares were collected from 2015 to 2017 in the province of Grosseto (central Italy). 37 hares (27 females and 10 males; 7 young hares and 30 adults) were legally culled, according to Italian Law No. 157/92 and 33 hares were collected from two farms (20 females and 13 males; 7 young hares and 26 adults). All samples were taken to the Office for Hunting and Fishing in Grosseto.

The viscera were separated from the rest of the carcass by hunters or breeders, therefore in some cases not all organs of hunted hares were properly conferred. Conversely samples of all organs of bred hares were accurately collected and analysed. The samples were transferred to the Parasitology Section of the Department of Veterinary Sciences at the University of Pisa.

Individual data on the area of origin, gender, weight, and age of the hares were recorded. Hares were classified as young (≤ 8 months of age) if the Stroh's tubercle was present, otherwise they were classified as adult (>8 months of age) (Stroh, 1931).

The gastro-intestinal tracts of 31 hunted hares and 33 bred hares were analysed. Intestinal mucosa was observed macroscopically, gastric contents were submitted to sedimentation and examined under a stereomicroscope. Intestines were examined with the sedimentation and counting technique (SCT) in accordance with Eckert *et al.* (2001).

Kidneys, urinary bladders, and spleens of 31 hunted hares and 33 bred hares were opened, washed and the sediment was examined by stereomicroscopy.

The livers of 24 hunted hares and 33 bred hares were analysed by stereomicroscopy.

The cardiorespiratory system of 24 hunted hares and 33 bred hares was examined. The trachea was examined under a stereomicroscope, pulmonary tissue smears were taken to search for eggs and larvae. Heart and lungs were cut and washed with tap water in order to collect adult parasites.

All adult parasites found were isolated, counted, separated by gender and stored in 70 % alcohol and classified, according to taxonomic keys and some articles (Yamaguti 1959; Levine 1968; Soulsby 1968; Boev 1975; Durette-Desset, 1977; Audebert *et al.*, 2000).

Rectal faecal samples (at least 3g) of all 70 hares were subjected to coprological analysis to detect parasite oocysts, eggs and larvae, using flotation in centrifuge with 50 % $ZnCl_2$ (s.g. 1.300) and K_2HgI_4 (p.s.1450) as flotation solutions, according to the procedure described by Dryden *et al.* (2005).

Coprocoltures of fresh faeces of bred hares were performed to obtain the coccidian oocysts sporulation in order to allow the identification of the coccidia species. Faeces with oocysts were treated with K2Cr2O7 to enable oocysts to sporulate (Eckert *et al*., 1995).

Prevalences with 95 % confidence intervals (CI), mean abundance, mean intensity, and range were calculated (Bush *et al.,* 1997). Multiple parasite infections were also described. Pearson's Chi squared test and Fisher's exact test were carried out to compare parasite prevalences in hares of different age groups, genders and provenance (i.e. free-living vs. bred hares). For the most prevalent parasite, a negative binomial distribution was compared to obtained data by Pearson's Chi square test in order to investigate the stability of host-parasites relationship as an indication of overdispersion (Bliss & Fisher, 1953). The relationship between the intensities of parasitic infections as predictor variables and body weight as a dependent variable was evaluated using Pearson correlation and the results were expressed as correlation coefficients and regression equations.

The results of the coprological tests were compared to necropsy results taken as a gold standard. The significance of the tests was confirmed for P values lower than 0.05. All the statistical analyses were carried out using Quantitative Parasitology 3.0 software (Rózsa *et al.,* 2000) and SPSS package version 20.

Ethical Approval and/or Informed Consent

The present work did not involve the use of laboratory animals. Samples were gathered from dead free-ranging brown hares which were legally shot by hunters in accordance with Italian Law (157 of 11/02/1992). Thus, no animals were killed specifically for this study.

Results

Hunted hares

The most prevalent parasites in the intestines of hunted hares were *Trichostrongylus retortaeformis* (87.1 %) in the small intestine, followed by *Passalurus ambiguus* (12.9 %) in the large intestine, and Cestoda of the family Anoplocephalidae (9.6 %) in the small intestine. On the basis of morphologic and morphometric analyses of scolex and proglottids, the cestodes were identified as *Andrya* spp. (Table 1).

Of the 31 examined hares, 12.9 % were negative to parasites, 67.7 % were positive to one parasite species, and 19.4 % presented double infections. All single infections were by *T. retortaeformis*, double infections were by *T. retortaeformis* and *P. ambiguus* (12.9 %), and by *T. retortaeformis* and *Andrya* spp. (6.5 %).

Comparing prevalences of intestinal parasites between genders, no significant differences were found. Between age classes, a sig-

Table 1. Necropsy of free-living hares *Lepus europaeus*. Intestinal and liver helminths. NP = number of positive hares, P% = Prevalence, CI %=95% confidence interval of prevalence, TP = total number of parasites, R = Range, A = Abundance; MI = Mean Intensity.

Necropsy	NP	P (%)	CI(%)	R	TP	A	IM
Hunted hares (n=37)							
Intestinal parasites[a]							
Trichostrongylus retortaeformis	27	87.1	70.1 – 96.3	2 – 3650	18505	596.9	685.4
Passalurus ambiguus	4	12.9	3.6 – 29.8	23 – 664	793	25.6	198.2
Andrya spp.	2	6.4	0.8 – 21.4	1 – 2	3	0.1	1.5
Liver parasites[b]							
Dicrocoelium dendriticum	4	16.7	0.8 – 21.4	16 – 610	644	26.8	161.0
Bred hares (n=33)							
Intestinal parasites							
Trichostrongylus retortaeformis	1	3.0	–	–	79	2.4	79
Passalurus ambiguus	4	12.1	3.4 – 28.2	1 – 312	370	11.2	92.5

[a]The gastro-intestinal tracts of 31 hunted hares were analysed.
[b]The livers of 24 hunted hares were analysed.

nificant difference was observed only for *T. retortaeformis* (P value of Fisher's exact test = 0.008): adult hares (25/26 hares, 96.2 %) were more infected than young hares (2/5 hares, 40.0 %).
T. retortaeformis was found with a high abundance and high intensity (597 and 685 respectively), with a high total number of parasites isolated (18505), ranging from 2 to 3650 specimens.
For *T. retortaeformis*, a negative binomial distribution, with parameters k = 0.44 and p binom = 0.00074, was found to fit the data and tested (Pearson's Chi squared P value higher than 0.05). No correlation was found between body size and the intensity of infection of *T. retortaeformis* ($r = 0.291$; $p = 0.119$).
No parasites were found in the spleen nor in the urinary system.
The most prevalent parasite in the bile ducts was *Dicrocoelium dendriticum* found with a prevalence of 16.7 %. No significant differences in the prevalences between genders and age classes were observed.
In the cardiopulmonary system, no parasites were found in the hearts and tracheas of the examined hares. In the lungs of two hares, parasites of the family Protostrongylidae were found, species *Protostrongylus cuniculorum*. The nematodes were clustered in nodules on the lung surface. Due to the difficulty of isolating parasites from nodules, it was only possible to calculate the prevalence of the infection and not the number of parasites. In addition to adult parasites, the presence of larvae was evidenced by pulmonary smears. The prevalences were not significantly different between genders and age classes of the hosts. The species identification was established on caudal bursa according to the features proposed by Boev (1975).
Coprological analysis revealed that 30 faecal samples were positive to some parasites (Table 2).
The results of the coprological test were compared to necropsy results (considered as the gold standard) in 31 hunted hares for the most prevalent parasite *Trichostrongylus retortaeformis*. The sensitivity (s) of the coprological test was 0.33 and the specificity (s') was 1.00.
A total of 24 free living hunted hares were positive to coccidia (64.9 %). There were no significant differences in the prevalences between age classes and genders. No correlation was found between body size and the intensity of infection of coccidia ($r = -0.160$; $p = 0.179$).

Table 2. Coprological examination hares *Lepus europaeus*. NP = number of positive hares, P%=prevalence, CI%=95% confidence interval of prevalence, R= Range, Mean OPG/EPG=Mean oocysts/eggs per gramme of faeces.

Coprology	NP	P%	CI%	R	Mean OPG/EPG
Hunted hares (n=37)					
Coccidia	24	64.9	47.5 – 79.8	50 – 26600	4765.2
Gastro-intestinal Strongylids	11	29.7	15.9 – 47.0	50 – 200	59.1
Dicrocoelium dendriticum	4	10.8	3.7 – 25.4	100 – 600	175
Bred hares (n=33)					
Coccidia	15	45.4	28.1 – 63.6	150 – 51750	5037.8

Table 3. Epidemiological studies on the prevalences of parasites in hares in Europe.

Country	EL[a]	PL[b]	AT[c]	CZ[d]	SK[e]	ES[f]	FI[g]	FR[h]
N° of hares	84	83	225	137	74	53	24	22
Trichostrongylus retortaeformis	50	32.5	82.7	83.2	6.8	56.6	54.2	9
Passalurus ambiguus	4.8	6.0	--	--	12.2	--	--	30
Protostrongylus spp.	1.2	4.8	37.3	18.2	--	--	33.3	--
Andrya spp.	--	--	4.4	2.9	--	34	--	--
Dicrocoelium dendriticum	9.5	--	--	--	--	11(7/64)	--	--
Coccidia	64.3	--	80.4	79.6	92	71.7	37.5	--

[a] Greece - Diakou *et al*., 2014; [b] Poland - Kornas *et al*., 2014; [c] Austria - Chroust*et al*., 2012; [d] Czech Republic - Chroust*et al*., 2012; [e] Spain - Dubinsky *et al*., 2010; [f] Finland - Alzaga *et al*., 2009; [g] Slovakia - Soveri & Valtonen, 1983; [h] France - Bordes *et al*., 2007;

Bred hares

On farms, necropsy examination of the large intestine, revealed the presence of *P. ambiguus* in four hares (12 %) with relatively low abundances and *T. retortaeformis* in one hare (3 %). All other organs were free from parasites (Tab. 1). Coprology examination revealed that 15 hares were positive only to the coccidia genus *Eimeria* spp., sometimes also with cases of large infection, with the OPG ranging between 150 – 51750 (Tab. 2). Three species of coccidia were identified in the bred hares: *Eimeria leporis*, *E. semisculpta*, and *E. europaea*.

Free-living vs. bred hares

A comparison between free-living and bred hares showed significant differences in the prevalences of *T. retortaeformis* (P value of Fisher's exact test < 0.001), and *D. dendriticum* (P value of Fisher's exact test = 0.03). For each parasite above mentioned, free-living hares (29.7 %; 10.8 %) were more infected than bred hares (3 %, 0 %).

Discussion

The intestinal helminths found in hunted hares were the nematodes *Trichostrongylus retortaeformis* and *Passalurus ambiguus*, and the cestode *Andrya* spp.

T. retortaeformis (87 %, 27/31), the dominant parasite in the present study, is a commonly encountered species in the small intestine of the European hare in Italy and in other areas of Europe. With regard to previous studies in other parts of Italy, a prevalence of 72 % (38/53) was found in Bologna (Stancampiano *et al.,* 2016), where it was the only gastro-intestinal strongylid detected. In Genoa the prevalence was 65 % (34/52) (Poglayen *et al.,* 2002), corresponding to the lowest value in Italy. In central Italy the prevalence was 75 % (54/72) (Poli *et al.,* 1991). Canestri-Trotti *et al.* (1988) compared the prevalence of this helminth in autochthonous and imported European hares. They found a prevalence of 71.9 % (59/82) in Ferrara, 90 % (29/30) in Modena, and 100 % (25/25) in hares imported from Czechoslovakia, Poland and Hungary. In the province of Pisa, a prevalence of *Trichostrongylus* spp. of 76.25 % (22/29), accompanied by catarrhal enteritis was reported by Agrimi *et al.* (1981).

Considering results obtained in other European countries, we found prevalences similar to those detected in Austria and Czech Republic, while elsewhere *T. retortaeformis* prevalences were lower (Table 3).

We found that the distribution of *T. retortaeformis* in the host population had a negative binomial distribution, indicating a good ecological balance and equilibrium between parasites and hosts. The distribution pattern of parasites in host population has many involvements in epidemiologic studies and host-parasite dynamics (Anderson & May, 1978; Poulin, 1993). For macro-parasites, host morbidity and mortality are strictly density-dependent and the effects will be high when parasites follow a random distribution (with a low level of variance) in host population. Consequently, an aggregate distribution (with high level of variance) positively affects the impact of parasite in host population supporting a stable interaction (Anderson & May, 1978; May & Anderson, 1978). The prevalence of the parasite was significantly higher in adults than in young hosts, in accordance with other studies (Stancampiano *et al.,* 2016).

The importance of this nematode as a pathogenic agent that induces mortality or weight loss has been demonstrated by Gottschalk (1973), Haupt & Hartung (1977) and by Boch & Schneidawind (1988). Newly *et al*. (2005) reported that *T. retortaeformis* infection involves a strong reduction in the physical condition and fertility of females, but does not affect their survival. However, our results suggest that the intensity of parasitic infection not affect hare body size.

P. ambiguus was found in 12.9 % of hares (4/31), higher than in other studies performed in Italy. In Genoa, the percentage was 3.8 % (2/52) (Poglayen *et al.,* 2002). Percentages of 7.3 % (6/82) and 10.0 % (3/30) were found in the provinces of Ferrara and Modena respectively, while the parasite was not found in 25 imported hares (Canestri-Trotti *et al.,* 1988).

The nematode, specific to lagomorphs, lives in the cecum and anterior colon and usually does not show clinically evident signs

(Diakou *et al.*, 2014). It is rarely found in European hares and always with low prevalences, while it is more commonly found in wild rabbits (*Oryctolagus cuniculus*).
In Europe *P. ambiguous* had higher prevalences only in France (Table 3).
Andrya spp.was found in this study, with a low prevalence 6.4 % (2/31). Previous studies in Italy showed the presence of the *Andrya* genus in Ravenna (Zanni *et al.,*1995). *A. rhopalocephala* and *Cittotaenia pectinata* were found in Pisa (Poli *et al.,* 1988). *A. rhopalocephala* was found in 3/30 hares (10 %) in Modena and in 1/25 imported hares (4 %) (Canestri-Trotti *et al.,* 1988). *Andrya cunicoli* was found in 9/137 imported hares (6.5 %) in Ferrara and Modena (Canestri-Trotti*et al.,* 1988).
In Europe *Andrya* spp. had higher prevalences only in Spain (Table 3).
Anoplocephalidae infestation generally does not cause any symptoms, only in case of massive infestations digestive disorders can occur (Heintzelmann-Grongroft, 1976).
In the present study *P. cuniculorum* was found in 8.3 % (2/24 hares). *P. pulmonalis* is the most common pulmonary parasite in Italy and Europe. However, in some locations in Tuscany the parasite *P. cuniculorum* (Joyeus & Gaud, 1946) was found in hares with pulmonary protostrongylosis (Barbiera, 1960).
P. rufescens and *P. pulmonalis* were found in some repopulation and hunting areas in Pisa (Poli *et al.,*1988). Furthermore, *Protostrongylus* spp. was found in 2.7 % (2/72) hares in the same area (Poli *et al.,*1991). *Protostrongylus* spp. was found in *L. europaeus* with a prevalence 14 % in Ravenna (Zanni *et al.,*1995).
In Europe *Protostrongylus* spp. prevalences are very variable (Table 3).
The presence of pulmonary stronglyes often predisposes to secondary bacterial infections, leading to alterations in lung capacity and function, which considerably reduce the possibility of escape of the hare and therefore its chances of survival (Spagnesi &Trocchi, 1992).
Dicrocoelium dendriticum was found in four livers of the 24 examined (16.6 %). *Dicrocoelium* spp. is mainly widespread among ruminants in pasture, but little known and often underestimated (Otranto & Traversa, 2002). Hares may occasionally be infected in areas of ruminant grazing (Chroust *et al.,* 2012). *D. dendriticum* was found for the first time in hares in Italy in one European hare, with prevalence 1.3 % (1/72) (Poli *et al.,* 1991).
It has also rarely been found in other areas of Europe (Table 3).
Coccidia had similar prevalence among our samples: 64.9 % (24/37) in hunted hares and 45.4 % (15/32) in bred hares. Genoa has the lowest prevalence (17.3 %, 9/52) (Poglayen *et al.,* 2002). Tacconi *et al.,* (1995) reported that in Umbria all faecal specimens of bred hares were negative for coccidia (0/867), while all free living hares in protected areas were positive (n = 1233). In central Italy, a prevalence 90.3 % (65/72) was found (Poli *et al.,* 1991). Coccidiosis was also found in the province of Pisa in 26.2 % (8/29) of dead hares (Agrimi *et al.,* 1981).
In Europe coccidia prevalences are generally high (Table 3).
Coccidia are among the most dangerous pathogenic parasites in hares. The combination of intestinal nematodes and coccidia are one of the major controlling factors in harbour populations (Chroust, 1984). Especially in young animals, a high level of infection can lead to severe illness and eventually to the death of the animal. However, our results suggest that the intensity of parasitic infection not affect hare body size.
By comparing the parasitic fauna of the free hares with the bred ones, the free hares were statistically more infected by parasites. These results could be expected due to the veterinary control of bred hares and the absence of intermediate hosts for the pulmonary strongyloidiasis. Furthermore, the risk of contracting bacteria and viral infections and parasites could increase based on the type of breeding farm (e.g. extensive or intensive farming) and represent an important limiting factor (Spagnesi & Trocchi, 1992).

Conclusion

Since the 1960s, the decrease in hare populations in Europe has led to management actions aimed at halting this reduction (Marboutin *et al.*, 2003). Fluctuations in the hare population have been attributed to many factors. However, the deterioration of animal health, strongly influenced by anthropization of the landscape, seems to be the key factor. The population density may also be influenced by various diseases, as well as by parasitic infections (Alzaga *et al.*, 2009). The results of the present study in the Grosseto area indicate that free-living hares have few species of parasites and that the intensities of parasitic infection did not affect their general condition and health, suggesting that endoparasites played no detectable role in the dynamics of this hare population. The aggregation of the most abundance parasite found (i.e. *T. retortaeformis*) suggests that only a minimal part of host population would, if ever, be influenced by this infection.
The situation observed in our study area, and in particular low parasite diversity, is consistent with the low host density (9 hares/ km2), that probably makes parasite transmission more difficult. The critical situation of host population parallels with the low biodiversity observed in parasite community. This could eventually induce a harmful loop, since biodiversity is considered a stabilizing factor in ecological webs and the lack of specific brown hare parasites in biocoenosis may be a predisposing factor for the occurrence of exogenous and potentially dangerous parasite *taxa* in the hare population (Hudson *et al.*, 2006). Parasite community has probably suffered, directly (environmental mechanisms acting on both host and parasites) or indirectly (mechanisms acting on host density and therefore on parasite transmission), the same unknown cause of brown hare decline.
Nevertheless, the good health status of the bred hares highlights a good health management of the farms. However, an in-depth analysis of the coccidia species is needed, as they had the highest prevalences in the sample studied.

Conflict of interest

Authors state no conflict of interest

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Helminth fauna of Valentin's Lizard *Darevskia valentini* (Boettger, 1892) (Squamata: Lacertidae) collected from central and eastern Anatolia, Turkey

S. BİRLİK[1*], H. S. YILDIRIMHAN[1], Ç. ILGAZ[2,3], Y. KUMLUTAŞ[2,3]

[1]Uludağ University, Faculty of Arts and Sciences, Department of Biology, Nilüfer, Bursa, Turkey, E-mail: *sezen@uludag.edu.tr*, *yhikmet@uludag.edu.tr*; [2]Dokuz Eylül University, Faculty of Science, Department of Biology, 35160, Buca-İzmir, Turkey, E-mail: *cetin.ilgaz@deu.edu.tr*, *yusuf.kumlutas@deu.edu.tr*; [3]Dokuz Eylül University Fauna and Flora Research Centre, 35610, Buca-İzmir, Turkey

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Summary

In this study, we assessed the helminth fauna of seventy two Valentin's Lizard, *Darevskia valentini* (32♂♂, 35♀♀, 5 subadult). Specimens collected from Kayseri, Ardahan and Van Provinces in Turkey. As a result of the present study, it was detected that forty one hosts are infected with one or more species of helminth. Two species of Cestoda, *Oochoristica tuberculata* and *Mesocestoides* spp., and 5 species of Nematoda, *Spauligodon aloisei*, *Skrjabinodon alcaraziensis*, *Skrjabinodon medinae, Skrjabinelazia hoffmanni* and *Strongyloides darevsky* were found in the hosts. *D. valentini* represents a new host record for all helminths recorded. *Skrjabinodon alcaraziensis* is recorded for the first time from Turkey. Van, Kayseri and Ardahan are new locality records for all helminths from *D. valentini*.
Keywords: *Darevskia valentini*; Cestode; Nematode; Turkey

Introduction

Darevskia valentini is present in west and central Anatolia, Turkey, southwestern Armenia and southwestern Georgia. Within the Caucasus it is found as isolated populations in Armenia, Azerbaijan, southern Georgia and eastern Turkey. The most extensive areas of the distribution range in the Caucasus are the high mountain areas of the Gegamsky range to the shores of Lake Sevan in the north, the high mountain area of the Aragaz Peak, and the high mountain plateau in the foothills of the Childyrsky and Javakhetsky ranges in the extreme north-west of Armenia and adjoining regions of southern Georgia. It is also found on the Ardenissky mountain range, and further east it inhabits the high-mountains of the Karabakh Upland within the limits of Nagorny Karabakh. The species is widely distributed in north-eastern, eastern and south-eastern Turkey. It is probably found in the high mountains adjacent Iran, although this needs to be confirmed. It might have been introduced to Ohio in the United States. In Turkey it has been recorded from 1,300 to 3,000m asl (Ananjeva *et al.,* 2006; Tok *et al.,* 2009; Baran *et al.,* 2012).

Helminths are parasites that infect internal and external organs of most invertebrate and vertebrate groups (Lima *et al.,* 2017). Knowledge of the lizard-associated helminth fauna has increased through research concerning (i) records of new hosts (Bursey & Goldberg 2004; Bursey *et al.,* 2005; Ávila & Silva, 2010; McAllister *et al.,* 2011; Ávila *et al.,* 2012), (ii) descriptions of new parasite species (Bursey *et al.,* 2003; Pereira *et al.,* 2012), and (iii) influence of biotic and abiotic variables on helminth diversity and abundance (Sharpilo *et al.,* 2001; Brito *et al.,* 2014a, b; Galdino *et al.,* 2014).

In Turkey, 66 lizard species occur (Baran *et al.,* 2012; Uetz, 2015); of these 66 species, only 30 have been studied from a helminthological point of view. Among this wide diversification of hosts, there is still less information on endoparasites of lizards in some parts of Turkey which makes understanding of the relationship between these parasites and their hosts difficult.

The current study characterizes the helminth richness of parasitic

* – corresponding author

species and the parameters of parasitic infection (prevalence, mean intensity of infection and range) in specimens of lizard collected from Kayseri, Ardahan and Van Proviences in Turkey and the aim of the present work is to enrich the existing knowledge about helminth parasites associated with Lacertidae, Turkey by analyzing specimens of this family found in Turkey.

Materials and Method

Mean snout-vent length of lizard specimens is = 62.31 ± 10.41 mm, with a range from 37.28 to 77.1 mm. All lizards were not in full sexual maturity (35 ♀♀, 32 ♂♂ and 5 subadults). We conducted the study in July and August in 2015 and 2016 at several localities of Kayseri, Ardahan and Van provinces, Turkey (Fig. 1). The hosts were dissected under a stereomicroscope and their body cavity, lung, stomach, small intestine and large intestine was analyzed in search of helminths. They were placed in separate petri dishes with 9 % saline solution and carefully dissected further under a stereomicroscope. The body cavity was also inspected for parasites. Helminths were removed from the gastrointestinal tract, counted, rinsed in saline and fixed with different hot solutions, depending on the type of parasite: nematodes were fixed in 70 % ethanol and cestodes were fixed in 70 % ethanol or acetic acid. All fixed worms were stored in 70 % ethanol until identified. For taxonomic identification, nematodes were cleared with glycerine: ethanol (2:1); cestodes were stained with acetocarmin.
Prevalence, mean intensity and abundance of infection of each helminth species were calculated as suggested by Bush *et al.* (1997). All helminths were identified under a light microscope according to the figures and descriptions given by Anderson (2000), Anderson *et al.* (2009), Yorke and Maplestone (1926), Schad *et al.* (1960), Yamaguti (1961), Schmidt (1986), Petter and Quantin (1976), Hughes (1940), Skryabin (1991) and Wittenberg (1934).
Finally, representative helminth specimens were deposited in the helminth collection of Uludağ University Museum of Zoology, Bursa, Turkey. Lizard specimens were deposited in the Dokuz Eylül University Fauna and Flora Research Centre, İzmir, Turkey.

Ethical Approval and/or Informed Consent

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. The study protocol no. 2013-04/08 was approved by Republic of Turkey, Uludag University Experimental Animals Local Ethics Committee**.**

Results

Seventy two lizards were captured and studied: *Darevskia valentini.* A total of seven species of helminths were recovered comprising two species of cestodes and five species of nematodes. The cestodes recovered include; *Oochoristica tuberculata* and *Mesocestoides* sp. The nematodes recovered were *Skrjabinelazia hoffmanni*, *Skrjabinodon medinae*, *Skrjabinodon aloisei*, *Spauligodon aloisei* and *Strongyloides darevsky* with prevalence of 58.33 %. The helminths recovered from lizards examined from Kayseri, Van, Ardahan provinces and their prevalences, mean abundance and intensity are given in Table. 1.

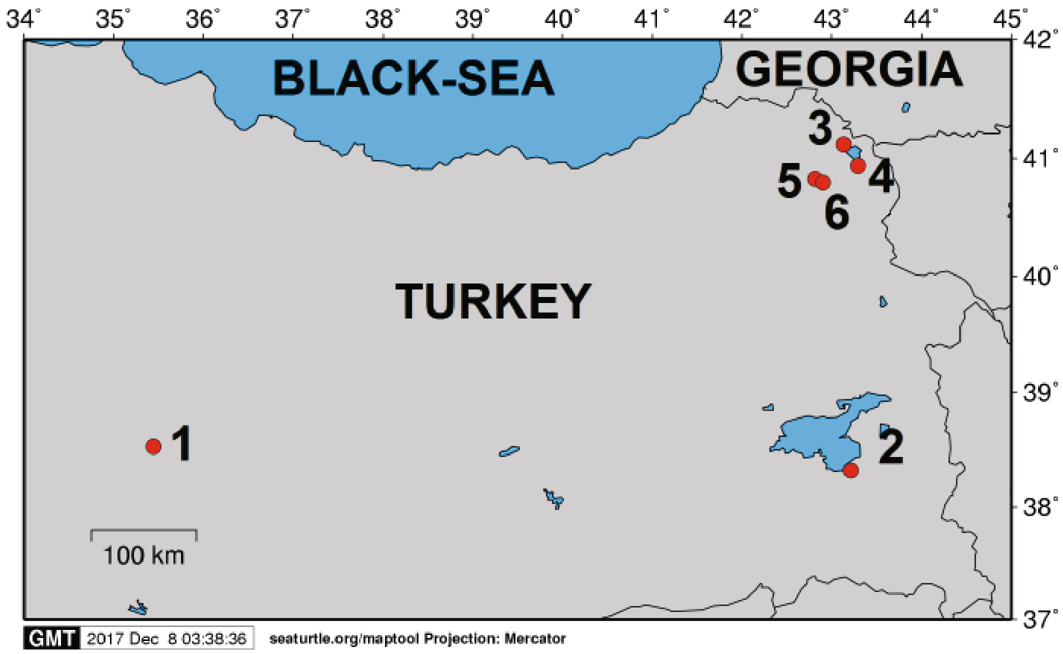

Fig. 1. The localities of host populations of *Darevskia valentini* (1. Erciyes Mountain, Kayseri; 2. Between Van-Bahçesaray 79. km; 3. Between Göle-Susuz 17. km Ardahan 4. Çıldır Lake, Ardahan 5. Çıldır-Taşbaşı Village, Ardahan; 6. Between Susuz-Göle 35. km Ardahan).

Table 1. Prevalence, mean intensity and mean abundance.

Helmint Species	Site of infection	Prevalence (%)	Mean intensity	Mean abundance
Cestoda				
Mesocestoides sp.	Intestine	5.55	too numerous to count	too numerous to count
Oochoristica tuberculata	Intestine	2.77	9	0.25
Nematoda				
Skrjabinodon medinae	Small intestine	15.27	3.9	0.59
Skrjabinodon alcaraziensis	Small intestine	1.38	1	0.013
Spauligodon aloisei	Small intestine	30.55	5.27	1.61
Strongyloides darevsky	Small intestine	2.77	1	0.027
Skrjabinelazia hoffmanni	Small intestine	9.72	3.14	0.3

Fifty-seven percent of hosts were parasitized by at least 1 species of helminth. In total 202 individuals of seven parasites species were collected from 42 of the 72 Valentin's Lizards examined. Of all the lizards analyzed, thirty were not parasitized. Of the infected lizards, 33 harbored one species of helminth, 4 harbored two species, 2 harbored three species and 2 harbored four species.

Discussion

Helminth species recorded in this study primarily infect lizards through host diet, which coherent with previous studies.

In the present study, we recorded the first occurrence of all species parasitizing *D. valentini*. All species recorded here except one were reported in other studies carried out in Turkey. This suggests that these parasites have wide range in geographical distribution and especially Oxyuroid family members of Nematoda frequently found in Lacertid lizards.

The genus *Oochoristica* was established by Luhe (1898) for unarmed cestodes with irregularly alternating genital openings and a uterus that transmutes quickly in a way that eggs can be found embedded in the parenchyma of gravid segments. It comprises cestodes parasitizing reptiles and is cosmopolitan in its distribution. Within this group, which includes about 88 species (Bursey *et al.,* 2010) and more species have been added recently (Mašová *et al.,* 2010; Schuster, 2011; Bursey & Goldberg, 2011; Mašová *et al.,* 2012; McAllister & Bursey, 2017) about 17 species are known from Palearctic. *Oochoristica tuberculata* (Rudolphi, 1819) was chosen as type species. This is the fifth record of *O. tuberculata* in Turkey, other reports are from *Paralaudakia caucasia* (Yıldırımhan *et al.*, 2006), *Chalcides ocellatus* (İncedogan *et al.*, 2014), *Eremias suphani* (Düşen *et al.*, 2015) *Apathya cappadocica* (Birlik *et al.,* 2015) and *Acanthodactylus harranensis* (Düşen *et al.,* 2016). The total number of cestodes was 18.

Mesocestoides spp., this group of cestodes has unique and distinct characteristics among cyclophyllidean cestodes such as the median ventral position of the genital atrium and bipartite vitelline gland. It is found in different hosts (birds and carnivore mammals) in different geographic regions (Gallas & Silveria, 2011). In our study, we analysed of the gastrointestinal contents of lizards and found this species. These data suggest that reptiles could be the possible intermediate hosts of *Mesocestoides* spp. This is the sixth record of *Mesocestoides* spp. from Turkey. Other reports are from *Anatalocerta danfordi* (Gürelli *et al.,* 2007), *L. trilineata* (Yıldırımhan *et al.,* 2011), *A. cappadocica* (Birlik *et al.,* 2015), *P. laevis* (Birlik *et al.,* 2016) and *D. rudis* (Birlik *et al.,* 2018). *D. valentini* is a new host record for tetrahyridia of *Mesocestoides* spp.

Skrjabinodon alcaraziensis is recorded for the first time from Ardahan, Turkey. This species was identified first time from *Algyroides marchi* (Sauria: Lacertidae), Spain (Lafuente & Roca, 1995). We found one male specimen of this species (prevalence 1.38 %). Hornero and Roca (1992) stated that this species exhibits an unpaired papilla between the postcloacal pair.

Skrjabinodon medinae; This species was described by Garcia-Calvente (1948) as *Pharyngodon medinae*. It lacks caudal alae and possesses a single pair of sessile pre-cloacal papillae. According to Petter and Quentin (1976), in the Oxyuroid family Pharyngodonidae Travassos, 1919, the presence or the absence of caudal alae in the males, in conjunction with other characteristics, such as a sclerotised tri-valvulate oesophageal bulb and the position of the vulva, permit four genera to be distinguished: *Pharyngodon*, *Spauligodon*, *Skrjabinodon* and *Parathelandros* (Hornero and Roca, 1992). These species which belongs these genera are frequently found in lizards of family Lacertidae. This is the fifth report of *Sk. medinae* from our country. Others: *Lacerta trilineata* (Yıldırımhan *et al.,* 2011), *Apatyha cappadocica* (Birlik *et al.,* 2015), *Phoenicolacerta laevis* (Birlik *et al.,* 2016) and *Darevskia rudis* (Birlik *et al.,* 2018).

Spauligodon aloisei; previously, it was reported from *Iranolacerta brandthii* (Birlik *et al.,* 2017) and *Darevkia rudis* (Birlik *et al.*, 2018). This study is the third record of the species in Turkey. This species was identified first time from *Podarcis siculus* (Lacertidae) from Italy. It has spined tail both males and females and absence of a spicule in males. Genital pore and vulva are above the level of the oesophagal bulb.

Skrjabinelazia Sypliaxov, 1930 (Seuratoidea) parasitizes some families of saurians, mainly Gekkonidae and Lacertidae, and has world-wide distribution. Several species are present in the Palearctic region. They are described from restricted geographic areas. Worms are reported from the stomach as well as from the small and large intestine. Males are rare probably short-lived, since several species are known only by the females (Lhermitte *et al.,* 2008). *Skrjabinelazia hoffmanni*; we have already found 20 female and 2 male specimens of this species from Erciyes Mountain, Kayseri. This is the fourth record of this species in Turkey, other reports are from *Anatololacerta danfordi* (Gürelli *et al.,* 2007)*, D. rudis* (Roca *et al.,* 2015a; Birlik *et al,* 2018).

Strongyloides darevsky, the genus *Strongyloides* Grassi, 1879 includes a great number of species parasitizing different amphibians, reptiles, birds and mammals (Roca and Hornero, 1992). Roca *et al.* (2016) stated that *Strongyloides darevsky* is in fact a true *Darevskia* specialist since it has been recorded only from species of this genus. This is the fourth record of *S. darevsky* in Turkey, other reports are from *D. rudis* (Roca *et al.,* 2015a; Birlik *et al.,* 2018)*, D. armeniaca* (Roca *et al.,* 2016). We found only two female specimens from Ardahan (prevalence 2.77 %).

Normally, larger individuals depend on higher quantities of food, which results in bigger home ranges (Watve and Sukumar, 1995) and host size has also been associated with increasing parasite intensity in other vertebrates but we did not found a significant association between host length and endohelminth abundance in *D. valentini*. The sex of the host had no influence on the overall prevalence of helminth infections in the lizard *D. valentini* examined in this study, as both sexes have the same prevalence of infection. This may be due to the fact that both sexes ingest similar diet. *Amo et al.* 2005 stated that both sexes seem to be susceptible to parasite infections as the prevalence and intensity of infection were similar but there are studies which have a significant difference in the overall intensity of helminth infection and the sex of the lizard. This can be correlate with number of studied specimens. More host specimens will be studied in terms of helminthological examination. Therefore, additional studies are necessary in the different biomes to determine the true helminth diversity of this family of lizards.

In summary, one new helminth record, seven new host records and new geographic locality records are documented in this study. The data presented here serve to increase the knowledge of the fauna of gastrointestinal parasites of *D. valentini* for the first time. However, further studies need to be conducted with a larger sample to better understand the infection patterns of this lizard in Turkey.

Conflict of Interest

Authors state no conflict of interest.

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Raillietiella mottae (Pentastomida: Raillietiellidae) parasitizing four species of Gekkota lizards (Gekkonidae and Phyllodactylidae) in the Brazilian Caatinga

V. F. LIMA[1*], S. V. BRITO[4], J. A. ARAUJO FILHO[2], D. A. TELES[2], S. C. RIBEIRO[3], A. A. M. TEIXEIRA[2], A. M. A. PEREIRA[1], W. O. ALMEIDA[1]

[1]Programa de Pós-Graduação em Bioprospecção Molecular, Departamento de Química Biológica, Universidade Regional do Cariri – URCA, Rua Cel. Antônio Luiz, 1161, Campus do Pimenta, 63105-000, Crato, Ceará, Brazil, E-mail: *vandeberglima@hotmail.com*; [2]Programa de Pós-Graduação em Ciências Biológicas (Zoologia), Departamento de Sistemática e Ecologia – DSE, Centro de Ciências Exatas e da Natureza – CCEN, Universidade Federal da Paraíba – UFPB, Cidade Universitária, Campus I, CEP 58059-900, João Pessoa, Paraíba, Brazil; [3]Instituto de Formação de Educadores - IFE, Universidade Federal do Cariri - UFCA, Campus Brejo Santo, CEP 63260-000, Brejo Santo, Ceará, Brazil; [4]Centro de Ciências Agrárias e Ambientais, Universidade Federal do Maranhão – UFMA, Boa Vista, CEP 65500-000, Chapadinha, Maranhão, Brazil

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Summary

We tested the role of sex, size, and mass of the lizards *Phyllopezus pollicaris*, *Gymnodactylus geckoides*, *Hemidactylus agrius*, *Lygodactylus klugei*, and *Hemidactylus brasilianus* on the rates of pentastomid infection in the Brazilian Caatinga. We collected 355 individuals of these five species, of which four (prevalence of infection: *P. pollicaris* 15.9 %, *G. geckoides* 1.4 %, *H. agrius* 28.57 %, and *H. brasilianus* 4.16 %) were infected by *Raillietiella mottae*. Parasite abundance was influenced by host body size and mass only in *P. pollicaris*. Host sex did not influence the abundance of parasites in any species. *Hemidactylus agrius*, *G. geckoides*, and *H. brasilianus* are three new host records for pentastomids.
Keywords: Geckos; Pulmonary parasite; Semi-arid; South America

Introduction

Pentastomids are endoparasites of the vertebrate respiratory system, whose definitive or intermediate hosts are usually reptiles. There are five pentastomid species of the genus *Raillietiella* that parasitize lizards in the Neotropics: *R. cartagenensis* Ali Riley & Self, 1985, *R. freitasi* (Motta & Gomes, 1968), *R. furcocerca* (Diesing, 1863), *R. frenata* Ali, Riley & Self, 1981, and *R. mottae* Almeida, Freire & Lopes, 2008.

Previous studies found pentastomids infecting insectivorous lizards in Brazil. For example, one study found *Mabuya agilis* (Raddi, 1823) infected by *Raillietiella* sp. (Vrcibradic *et al*., 2002). Another study in a restinga (Almeida *et al*., 2009a) found *Cnemidophorus abaetensis* Dias, Rocha & Vrcibradic, 2002 and *C. ocellifer* (Spix, 1824) parasitized by *Raillietiella* aff. *furcocerca* (Almeida *et al*., 2009a) and *Micrablepharus maximiliani* (Reinhardt & Luetken, 1862) parasitized by *R. mottae* (Almeida *et al*., 2009a). Also, Anjos *et al*. (2007) and Almeida *et al*. (2008c) found *R. mottae* and *R. renata*, Sousa *et al*. (2014) recorded only *R. mottae* parasitizing *Hemidactylus mabouia* (Moreau de Jonnès, 1818) in houses, *R. frenata* in the north east coast of Brazil (Bezerra *et al*., 2016), while *Trachylepis atlantica* (Schmidt, 1945) was infected by *R. freitasi* in the Archipelago of Fernando de Noronha, Pernambuco (Brito *et al*., 2012). In the Caatinga, *R. mottae* was recorded infecting *Tropidurus hispidus* (Spix, 1825) (Almeida *et al*., 2008a, 2008b, 2009b; Brito *et al*., 2014b; Araujo Filho *et al*., 2016), *T. semitaeniatus* (Spix, 1825) (Almeida *et al*., 2008b, 2009b, Brito *et al*., 2014b), *Phyllopezus periosus* Rodrigues, 1986 (Almeida *et al*., 2008b; Brito *et al*., 2014b), *P. pollicaris* (Spix, 1825) (Almeida *et al*., 2008b; Sousa *et al*., 2010, 2014; Brito *et al*., 2014b) and *M. arajara* Rebouças-Spieker, 1981 (Ribeiro *et al*., 2012).

Regarding abundance of pentastomids in Brazil, previous studies,

* – corresponding author

found less than 14 % prevalence for pentastomids with at least 15 lizards collected from the same species. For example, *C. abaetensis* (n = 33, prev. = 6 %, Dias *et al.*, 2005), *C. ocellifer* (n = 40, Prev = 2.5 %), *M. agilis* (n = 28, Prev = 3.6 %, Vrcibradic *et al.*, 2002), *T. hispidus* (n = 18, Prev = 11.1 %, Almeida *et al.*, 2008a; n = 288, Prev. = 1 %, Brito *et al.*, 2014b; n = 411, Prev. = 0.66 %); *T. semitaeniatus* (n = 15, Prev = 13.3 %, Almeida *et al.*, 2008b; n = 120, Prev = 0.51 %, Brito *et al.*, 2014b); *M. maximiliani* (N = 75, Prev = 4 %, Almeida *et al.*, 2009a), and *M. arajara* (n = 125, Prev = 1.6 %, Ribeiro *et al.*, 2012). Only three studies with similar sample size (15 geckos) found a higher prevalence: *H. mabouia* (n = 37, Prev = 45.9 %, Anjos *et al.*, 2007; n = 30, Prev = 20 %, Almeida *et al.*, 2008c) and *P. pollicaris* (n = 22, Prev = 18.18 %, Sousa *et al.*, 2010).

Here we tested the role of host body size, mass and sex on the abundance of pentastomids in the insectivorous geckos *P. pollicaris* and *Gymnodactylus geckoides* (Spix 1825) (Phyllodactylidae), *H. agrius* Vanzolini, 1978, *Lygodactylus klugei* (Smith, Martin & Swain, 1977), and *H. brasilianus* (Amaral, 1935) (Gekkonidae) in a Caatinga area. These species were chosen because they have been reported to have higher prevalence in the literature.

Material and Methods

Lizard sampling and parasite identification

We sampled lizards at the Aiuaba Ecological Station (ESEC Aiuaba), municipality of Aiuaba, state of Ceará, northeastern Brazil (6° 36' 27" S; 40° 08' 00.9" W), which is within the Caatinga. The vegetation is composed of hypoxerophilic plants, shrubs, thorny trees, and open formations (Andrade-Lima, 1981). The climate is warm semi-arid tropical, with an average annual rainfall of 562.4 mm; the average temperature varies from 24 °C to 26 °C, with a short rainy period between February and April (IPECE, 2011).

We collected lizards using visual encounter surveys (McDiarmid, 2012) in September and November 2014 and February and April 2015. We measured Snout-Vent Length (SVL) with a digital caliper (to the nearest 0.01 mm), body weight with a digital scale (to the nearest 0.01 g). Then, we removed the specimens' respiratory tract under the stereomicroscope to inspect and count the parasites. Sex and sexual maturity were determined by analyzing the gonads. Mature males were those individuals with developed testes and epididymas with convolutions, and mature females were those with vitellogenic follicles in ovaries or oviducts. Lizards were killed with a lethal dose of lidocaine and fixed with 10 % formaldehyde and stored in 70 % ethanol.

Parasites were lightened in Hoyer medium (Everhart, 1957), mounted on temporary slides, and preserved in 70 % ethanol. Identification was conducted under microscope coupled with gridded ocular based on the size of mating hooks and spicules of males (Riley *et al.*, 1986; Almeida *et al.*, 2008a). Parasites will be deposited in the Parasitological collection of the Regional University of Cariri – URCA.

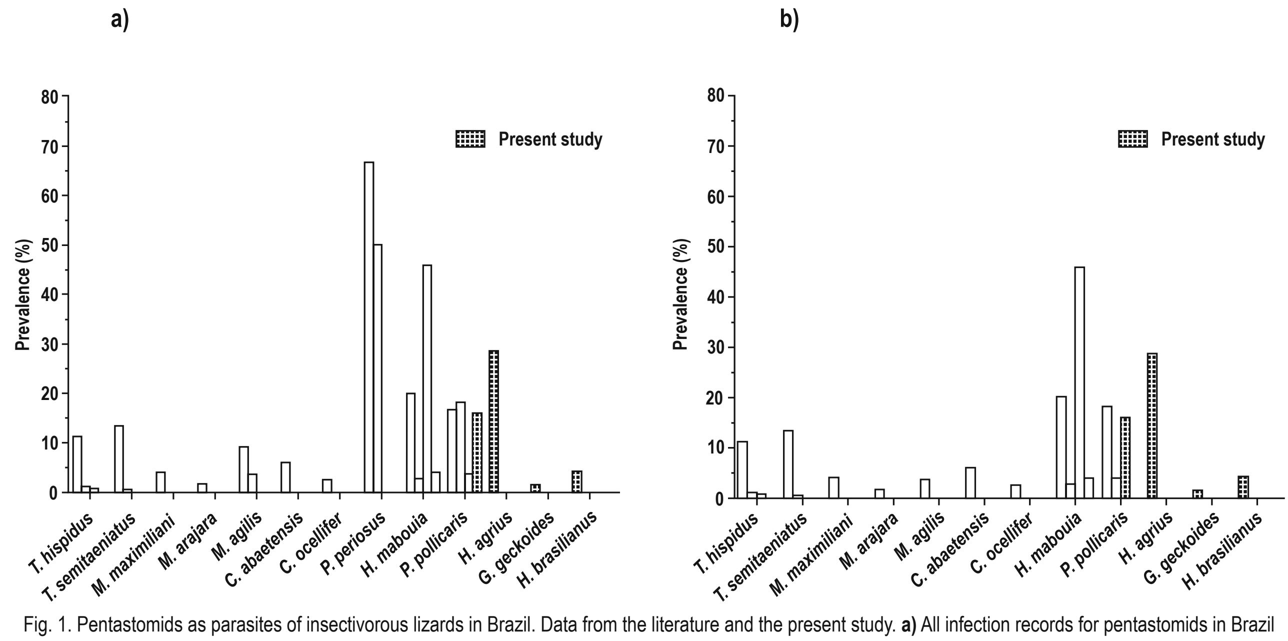

Fig. 1. Pentastomids as parasites of insectivorous lizards in Brazil. Data from the literature and the present study. **a)** All infection records for pentastomids in Brazil including this study, **b)** includes only studies that sampled more than 15 individuals of the same lizard species. References with number of hosts in parenthesis: *Tropidurus hispidus* (n = 18, Almeida *et al.*, 2008a; n = 288, Brito *et al.*, 2014b; n = 411, Araujo-Filho et al., 2016), *T. semitaeniatus* (n = 15, Almeida *et al.*, 2008b; n = 120, Brito *et al.*, 2014b), *Micrablepharus maximiliani* (n = 75, Almeida *et al.*, 2009a), *Mabuya arajara* (n = 125, Ribeiro *et al.*, 2012), *M. agilis* (n = 11, n = 28, Vrcibradic *et al.*, 2002), *Cnemidophorus abaetensis* (n = 33, Dias *et al.*, 2005), *C. ocellifer* (n = 40, Dias *et al.*, 2005), *Phyllopezus periosus* (n = 6, Almeida *et al.*, 2008b; n = 6, Brito *et al.*, 2014b), *Hemidactylus mabouia* (n = 37, Anjos *et al.*, 2007; n = 30, Almeida *et al.*, 2008c; n = 76, Sousa *et al.*, 2014; n = 277, Bezerra *et al.*, 2016), *P. pollicaris* (n = 6, Almeida *et al.*, 2008b; n = 22, Sousa *et al.*, 2010; n = 94, Brito *et al.*, 2014b; n = 132, this study) *H. agrius* (n = 63, this study), *Gymnodactylus geckoides* (n = 71, this study) and *H.brasilianus* (n = 24, this study).

Statistical analyses

We calculated the prevalence (the number of hosts infected by one or more parasites divided by total hosts number), abundance (the number of parasites of a given host whether or not it is infected), range (the number of parasites in a given host species, with a smallest number is given the number one, and other numbers represent the largest number of parasites found in an individual of the host species) and mean intensity of infection (the total number of parasites species found in a sample divided by the number of hosts infected with that parasite) for each lizard species following Bush *et al*. (1997). First, we tested whether there is a relationship between pentastomids abundance (response variable, log-transformed and using a Poisson response distribution) and SVL, sex, species and interaction between sex and species of lizards. Second, we tested whether there is a relationship between infection (presence/absence and using a binomial response distribution) and the explanatory variables, mass, sex, species and interaction between sex and species of lizards, the selected statistical test Generalized Linear Model (GLM) is efficient to avoid the confounding effect of host size (Poulin, 1997). We only used host species with more than ten individuals infected (at least five males and five females) in the analyses. Only *P. pollicaris* and *H. agrius* met this criterium, because *G. geckoides* and *H. brasilianus* presented only one lizard infected by pentastomids. All analyzes were performed using the Software R, package "R commander" (R core team, 2008) (Wilson & Grenfell, 1997).

Ethical Approval and/or Informed Consent

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

We collected 355 individuals from five lizard species. Of these, three species belonged to the family Gekkonidae *H. agrius*: 28 males (SVL = 47.07 ± 3.83 mm) and 35 females (SVL = 47.82 ± 3.78 mm); *L. klugei*: 25 males (SVL = 27.52 ± 2.58 mm) and 40 females (SVL = 28.02 ± 2.41 mm); and *H. brasilianus*: 11 males (SVL = 45 ± 5.93 mm) and 13 females (SVL = 43.61 ± 5.70 mm). And another two species belonged to the family Phyllodactylidae *P. pollicaris*: 57 males (SVL = 64.22 ± 8.40 mm) and 75 females (SVL = 62.58 ± 11.93 mm); *G. geckoides*: 30 males (SVL = 37.76 ± 3.70 mm) and 41 females (SVL = 37.53 ± 4.12 mm) (Fig. 1). The only parasite species found in four of the five lizard species was *Raillietiella mottae* (Table 1).

Body size and mass were significant for the abundance of pentastomids only for *P. pollicaris*, only when related by host sex. However, the SVL and mass only become significant in the part of the response variable (infected or uninfected host), regardless of the intensity of the infection (number of parasites per infected lizard). Thus, the SVL and mass determine whether or not the lizard will be infected, but not how many parasites it will harbor. Both sex and species were significant in both parts of the model, that is, these factors influence the infection and the amount of parasites (Table 2). We recorded three new hosts for *R. mottae*: *H. agrius*, *H. brasilianus*, and *G. geckoides*. There was no damage to the lung tissue of infected lizards.

Discussion

All lizard species found are of nocturnal activity and have a sit-and-wait foraging mode (Vanzolini, 1968). However, they differ in the strata used to forage. For example, *Hemidactylus agrius* and *P. pollicaris* are common in rocky outcrops, while *Hemidactylus brasilianus* and *L. klugei* are tree dwellers, and *G. geckoides* forages in the leaf litter (Vitt *et al*., 1995; Colli *et al*. 2003; Rocha *et al*., 2005; Mesquita *et al*., 2006; Sousa *et al*., 2010; Recorder *et al*., 2012; Albuquerque *et al*., 2013; Passos *et al*., 2014). The similar nocturnal activity of the species may render similar insect prey and allow the infection by *R. mottae*, which seems to be a generalist parasite,

Table 1. Total infection rates for each lizard species discriminated by sex in the Ecological Station of Aiuaba, Ceará, Brazil.

Species	*Raillietiella mottae*					
	Prevalence (%) Intensity (range)					
			Sex			
	Total		Male		Female	
Gekkonidae						
Hemidactylus agrius	28.57 %	1.66 (1 – 5)	39.28 %	1.27 (1 – 2)	20 %	2.28 (1 – 5)
Hemidactylus brasilianus	4.16 %	1 (1)	0	0	7.69 %	1 (1)
Phyllodactylidae						
Phyllopezus pollicaris	15.9 %	1.9 (1 – 5)	87.7 %	2 (1 – 5)	21.33 %	1.87 (1 – 3)
Gymnodactylus geckoides	1.4 %	5 (5)	0 %	0	2.43 %	5 (5)

Table 2. Generalized Linear Model (GLM) in Poisson distribution on the variation of the pentastomids abundance related to the host specie (*Phyllopezus pollicaris*), sex, mass, Snout-Vent Length (SVL) and interaction SVL, mass with sex × host species. Values of P presented in italics are statistically significant.

	Estimate	Std. Error	z value	P
Abundance ~ SVL	-0.062	0.035	-1.778	0.075
Abundance ~ Sex	-1.136	0.496	-2.292	*0.022*
Abundance ~ SVL * Sex	1.512	0.684	2.212	*0.027*
Abundance ~ Mass	0.344	0.072	4.790	0.167
Abundance ~ Sex	0.117	0.366	0.320	0.749
Abundance ~ Mass * Sex M	-1.080	0.517	-2.088	*0.037*

infecting several lizard species (Dias *et al*., 2005; Vrcibradic *et al*., 2002; Anjos *et al*., 2007; Almeida *et al*., 2008a, 2008b, 2008c, 2009a; Sousa *et al*., 2010, 2014; Ribeiro *et al*., 2012; Brito *et al*., 2014b; Araujo Filho *et al*., 2016).

However, there is no records of insects as intermediate hosts for *R. mottae*, even though Lavoipierre & Lavoipierre (1966) found *Periplaneta americana* as intermediate host of *R. hemidactyli* in Singapore. Almeida *et al*. (2008b) suggested that ants and termites could be intermediate hosts, because these insects are commonly found in lizard diets (Vitt *et al*., 1995, Colli *et al*., 2003, Rocha *et al*., 2005; Mesquita *et al*., 2006; Sousa *et al*., 2010; Recoder *et al*., 2012; Albuquerque *et al*. 2013; Passos *et al*., 2014) which could possibly contribute to infections.

The infected species (*H. agrius*, *H. brasilianus*, and *G. geckoides*) represent three new host records for *R. mottae*. We did not find lung lesions, probably because the maximum load of parasites in a single lizard was five. Pulmonary lesions in geckos are more frequent when they are infected by more than nine pentastomids (Riley *et al*., 1991).

The prevalence of *R. mottae* in Brazilian lizards varies according to the species, but it seems that only geckonids have a prevalence above 14 % (Anjos *et al*., 2007; Almeida *et al*., 2008c; Sousa *et al*., 2010; this study). Pentastomids may have a long co-evolutionary history with geckonids. This would also explain the small prevalence of *R. mottae* in other lizard lineages, even with large sample sizes (Dias *et al*., 2005; Vrcibradic *et al*., 2002; Almeida *et al*., 2008b; 2009a, Ribeiro *et al*., 2012, Brito *et al*, 2014b, Araujo Filho *et al*., 2016). These lizards would be occasional hosts due to opportunistic infections. Future studies are needed to confirm these hypotheses.

Host size and mass influenced the abundance of pentastomids only in *P. pollicaris*, mainly in males (only when related to host sex), but not in the amount of pentastomids per lizards (range). This relationship is identified here for the first time and is probably due to larger individuals having larger home ranges, having a more diverse diet of invertebrates, using a wider range of microhabitat, and being active longer. Thus this ratio of higher SVL and mass, represent higher niche and consequently greater abundance of pentastomides can be intensified in males of the host species in question. In addition the range might be influenced other factors not tested in the present study, possibly humidity, seasonality, temperature, among others. However, the same does not apply to larger lizards such as *Salvator merianae* and *Ameivula ocellifera* (Brito *et al*., 2014a; Ramalho *et al*., 2009; Teixeira *et al*., 2017).

The present study adds three new host records for *R. mottae*. Also, we found a positive relation between host size and mass in infection by pentastomids in lizards of the families Phyllodactylidae and Gekkonidae in the Caatinga. However, further studies are needed to better understand the ecological and co-evolutionary relationships between lizards and pentastomids in this biome.

Conflict of Interest

Authors state no conflict of interest.

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The diversity of teleost fish trematodes in the Bay of Bizerte, Tunisia (Western Mediterranean)

R. ANTAR*, L. GARGOURI

Unité de Recherche: Bio-Ecologie Animale et Systématique Evolutive, Faculté des Sciences, Université Tunis El Manar, 2092 Tunis, Tunisie, *E-mail: *rym.antar@hotmail.fr*

Article info

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Summary

A total of 39 digeneans species allocated to 28 genera in 12 families were recovered from 534 fishes belonging to 14 species in three families (Carangidae, Mullidae and Sparidae) collected in the Bay of Bizerte off the coast of Tunisia. We provide a host-parasite list of records from this locality, including 63 host-parasite combinations. The Opecoelidae Ozaki, 1925 is the most diverse group with 12 species. The species richness of individual digenean genera in the Bay of Bizerte ranges from 1 – 6 species. The mean number of 2.58 species per host indicates a relatively high digenean diversity in the Bay of Bizerte, which is related to its geographical location, its connection with the neighbouring Bizerte Lagoon and the nature of the bottoms of the littoral marine areas off the northern Tunisian coasts. This diversity is significantly higher than that reported off the southern coast of Tunisia and distinctly lower than that observed for teleost hosts in the Scandola Nature Reserve off Corsica. Generally, the levels of infection in teleosts fishes from the Bay of Bizerte are lower than those from the other two localities.
Keywords: Digenea; diversity; checklist; Bay of Bizerte; Mediterranean; Tunisia

Introduction

Parasites of aquatic organisms constitute an essential part of aquatic ecosystems. The evaluation of parasite biodiversity and the monitoring of community dynamics may constitute a good indicator of potential changes that might affect these ecosystems. These parasites include digeneans that often depend on trophic interactions for their transmission. Moreover, due to their complex life-cycles that require two or more intermediate hosts, digenean trematode parasites are linked to several different taxa and constitute excellent bioindicators of biodiversity (Marcogliese & Cone, 1997a, b; Hechinger *et al*., 2007). Positive correlations have been demonstrated between the digenean species richness and faunistic diversity (Hechinger & Lafferty, 2005; Hechinger *et al*., 2007). In the Mediterranean region, numerous studies, starting in the 19th Century, have been conducted on digenean species parasitic in fish. As a result, a total of 303 species has been reported and described from just 192 fish species (Pérez-del-Olmo *et al*., 2016; Rima *et al*., 2017). In contrast, in Tunisia, interest in these helminths has been developed only over the past 20 years with the pioneer studies of Gargouri Ben Abdallah and Maamouri (1997, 2002, 2005a, b, 2008) and Gargouri Ben Abdallah *et al*. (2010) on the diversity and the life-cycles of fish digeneans off the northern coast of the country. In 2012, another determined effort was made by Derbel and co-workers to contribute to our knowledge of the digenean fauna in marine fishes off the southeastern coast of Tunisia (Derbel *et al*., 2012). In an earlier study on the biodiversity of digeneans in the Bizerte Lagoon (an inlet of the Mediterranean in northeastern Tunisia), we reported 30 species of digeneans from sparid fishes (Gargouri Ben Abdallah *et al*., 2011; Antar & Gar-

* – corresponding author

gouri, 2013; Antar *et al*., 2015). Of these, two were recognised using both morphological and molecular methods (see Antar *et al*., 2015). In addition, the life cycle of *Proctoeces maculatus* (Looss, 1901) was studied in Bizerte Lagoon using the same methods, resulting in our suggesting the existence of cryptic species based on the different developmental stages of the life-cycle of this 'species' (Antar & Gargouri, 2015). In the present work, we provide information on the diversity of digenean species in teleost fishes off the northern coast of Tunisia (Bay of Bizerte) and compare our results with those already published for the other localities of the Mediterranean Sea.

Materials and Methods

Over a 3-year sampling period (2010 – 2012), a total of 534 teleosts was collected from the Bay of Bizerte (37°16'47.02"N 9°58'23.38"E) located off the northeastern coast of Tunisia. Fish were obtained fresh from local fishermen in the Bay of Bizerte, kept on ice and immediately brought back to the laboratory where they were identified based on Fischer *et al*. (1987), then measured, weighed, photographed and dissected. Fish names follow FishBase (Froese & Pauly, 2016). The gastrointestinal tract was then removed and examined for digeneans under a binocular microscope; the worms recovered were fixed alive by being pipetted into near-boiling saline. Afterwards, they were stained with borax carmine, dehydrated through a graded ethanol series, cleared in clove oil (eugenol) and examined as permanent mounts in Canada balsam. Several live specimens were examined for identification as temporary mounts in cold saline under the slight pressure of a coverslip. Ecological terms (prevalence, abundance and mean intensity) follow the definitions of Margolis *et al*. (1982).

Ethical Approval and/or Informed Consent

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

Results

A total of 39 digenean species allocated to 28 genera in 12 families were recovered from 534 fishes belonging to 14 different species from 12 genera in three families. Most of them were found in members of the Sparidae. We identified 63 host-parasite combinations (Table 1). The most species rich digenean family, the Opecoelidae Ozaki, 1925 (with 12 species), accounted for 30.8 % of the total species and included six species of *Macvicaria* Gibson and Bray, 1980, among which was *M. bartolii* Antar, Georgieva, Gargouri and Kostadinova, 2015, a species recently described using both morphology and molecular information from ITS1-5.8S-ITS2 sequences and partial sequences of the 28S rRNA gene (Antar *et al*., 2015), and one species each of *Allopodocotylle* Pritchard, 1966, *Opecoeloides* Odhner, 1928, *Pachycreadium* Manter, 1954, *Poracanthium* Dollfus, 1948, *Pseudopycnadena* Saad Fares and Maillard, 1986 and *Pycnadenoides* Yamaguti, 1938. The Opecoelidae is also the most diverse digenean family in the Mediterranean, with 42 species (Pérez-del-Olmo *et al*., 2016; Rima *et al*., 2017). The species richness of individual genera in the Bay of Bizerte ranged from 1 – 6 species, although most genera (78.6 %) have only a single species, which accounts for 22 of the digenean species. These genera exploit three infected fish families (Carangidae, Mullidae and Sparidae). After *Macvicaria*, *Holorchis* Stossich 1901 is the next most species rich genus, with three species.
Of the 14 teleost species examined, only *Pomatomus saltatrix* (Linnaeus, 1766) lacked digenean parasites. Two digenean species have been reported from this host in the Black Sea and off the southern coast of Tunisia (*Opechona bacillaris* (Molin, 1859) (see Bray & Gibson, 1990) and *Prosorhynchoides arcuatus* (Linton, 1900) (see Derbel *et al*., 2012)). The absence of digeneans in *P. saltatrix* in the Bay of Bizerte can be explained by the low numbers of this host examined due to its scarcity in the study area, its biological and ecological preferences and also by its small body size; the three specimens examined were all young. Several authors have highlighted the relationship between host body size and the number of parasites and their composition (Grutter & Poulin, 1998; Lo *et al*., 1998; Muñoz *et al*., 2002; Muñoz & Cribb, 2005, 2006). They concluded that larger host individuals had a higher species richness of parasites than smaller individuals. Indeed, resources are more important and microhabitats are more varied in a large rather than a small body (Muñoz *et al*., 2002; Muñoz & Cribb, 2005). The highest species richness was recorded in the sparid *Diplodus sargus sargus* (Linnaeus, 1758), with nine species (28 % of all species recorded in this study), followed by *Boops boops* (Linnaeus, 1758) and *Pagellus erythrinus* (Linnaeus, 1758), each with 7 species (21.9 %). The digenean faunas of these three fish species were dominated by *Macvicaria crassigula* (Linton, 1910), *Aphanurus stossichii* (Monticelli, 1891) and *Holorchis pycnoporus* Stossich, 1901, respectively. *Sarpa salpa* (Linnaeus, 1758) and *Sparus aurata* Linnaeus, 1758 each harboured six species (18.7 %), whereas *Mullus surmeletus* Linneaus, 1758 was infected with five trematode species (15.6 %). *Diplodus vulgaris* (Geoffroy St.-Hilaire, 1817) and *Pagrus pagrus* (Linnaeus, 1758) exhibited a lower diversity, not exceeding four digenean species (12.5 %). Five host species (9.4 %) (*Diplodus annularis* (Linnaeus, 1758), *Lithognathus mormyrus* (Linnaeus, 1758), *Oblada melanura* (Linnaeus, 1758), *Trachurus trachurus* (Linnaeus, 1758) and *Spondyliosoma cantharus* (Linnaeus, 1758)) were infected with three digenean species.
Examination of the parasitological descriptors of species richness (Margolis *et al*., 1982; Valtonen *et al*., 1997) shows that several species (e.g. *Arnola microcirrus* (Vlasenko, 1931), *Holorchis legendrei* Dollfus, 1946, *Macvicaria mormyri* (Stossich, 1885), *Magnibursatus bartolii* Kostadinova, Power, Fernández, Balbuena, Raga and Gibson, 2003, *Pycnadenoides senegalensis* Fischthal and Thomas, 1972, *Poracanthium furcatum* Dollfus,

Table 1. The prevalence (P), abundance (A) and mean intensity (MI) of digeneans collected from teleost fishes off the Bay of Bizerte, the Gulf of Gabes (Derbel *et al*., 2012) and Scandola Nature Reserve (Bartoli *et al*., 2005).

Locality				**Bay of Bizerte**			**Gulf of Gabes**			**Scandola Nature Reserve**		
References				**Present study**			**Derbel *et al*. (2012)**			**Bartoli *et al*. (2005)**		
Fish species (number of specimens)	**Family**	**Digenean species**	**Family**	**P (%)**	**A**	**MI**	**P (%)**	**A**	**MI**	**P (%)**	**A**	**MI**
Trachurus trachurus (Linnaeus, 1758) (n=15)	Carangidae	*Ectenurus lepidus* Looss, 1907	Hemiuridae	13.3	0.2	1.5	33	0.33	1	8	0.2	2
		Lasiotocus typicus (Nicoll, 1912)	Monorchiidae							8	0.1	1.5
		Monascus filiformis (Rudolphi, 1819)	Fellodistomidae	40	0.6	1.5	33	0.77	2.3	4	0.04	1
		Prodistomum polonii (Molin, 1859)	Lepocreadiidae							72	8.1	11.2
		Pseudopecoeloides chloroscombri (Fischthal & Thomas, 1970)	Opecoelidae	13.3	0.1	1				45.8	1	2.3
		Tergestia laticollis (Rudolphi, 1819)	Fellodistomidae							56	1.6	3.4
Mullus surmuletus Linnaeus, 1758 (n=16)	Mullidae	*Aponurus laguncula* Looss, 1907	Lecithasteridae							1.5	0.04	2.5
		Derogenes latus Janiszewska, 1953	Derogenidae							6.2	0.1	2
		Holorchis legendrei Dollfus, 1946	Aephnidiogenidae	6.2	0.06	1				13.8	0.4	2.8
		Lasiotocus mulli (Stossich, 1883)	Monorchiidae							27.7	5.4	19.6
		Lecithochirium musculus (Looss, 1907)	Hemiuridae							0.7	0.01	1
		Opecoeloides furcatus (Bremser in Rudolphi, 1819)	Opecoelidae	25	0.9	3.7	52.77	3.43	6.47	44.6	6	13.4
		Poracanthium furcatum Dollfus, 1948	Opecoelidae	6.2	0.1	2	13.88	0.40	3.57	50	14	28
		Proctoeces maculatus (Looss, 1901)	Fellodistomidae	6.2	0.06	1						
		Proctotrema bacilliovatum Odhner, 1911	Monorchiidae	43.7	29.4	67.1	20.83	1.46	7	33.1	4.1	12.3
		Stephanostomum sp.	Acanthocolpidae				9.72	2.91	30			
		Timonia mediterranea Bartoli & Prevot, 1966	Monorchiidae							10.8	1	9.2
Boops boops (Linnaeus, 1758) (n=143)	Sparidae	*Aphanurus stossichii* (Monticelli, 1891) Looss, 1907	Hemiuridae	30.1	1.4	4.6	33.33	1.38	4.1	84.6	12.6	14.9
		Arnola microcirrus (Vlasenko, 1931)	Derogenidae	0.7	0.01	1				30.8	0.9	3
		Bacciger israelensis Fischthal, 1980	Faustulidae	17.5	0.8	4.6	27.77	2	7.2	61.5	2.5	4.1
		Ectenurus lepidus Looss, 1907	Hemiuridae	0.7	0.01	1						
		Hemiurus communis Odhner, 1905	Hemiuridae	0.7	0.01	1				76.9	7.5	9.8
		Lepocreadium album (Stossich, 1890)	Lepocreadiidae	2.8	0.03	1.2						
		Robphildollfusium fractum (Rudolphi, 1819)	Gyliauchenidae							69.2	9	13
		Robphildollfusium martinezgomezi López-Román, Gijon-Botella, Kim and Vilca-Choque, 1992	Gyliauchenidae	2.1	0.03	1.7	5.55	0.16	3			

Locality				Bay of Bizerte			Gulf of Gabes			Scandola Nature Reserve		
References				Present study			Derbel *et al.* (2012)			Bartoli *et al.* (2005)		
Fish species (number of specimens)	**Family**	**Digenean species**	**Family**	**P(%)**	**A**	**MI**	**P(%)**	**A**	**MI**	**P(%)**	**A**	**MI**
Diplodus annularis (Linnaeus, 1758) (n=7)	Sparidae	*Diphterostomum brusinae* (Stossich, 1889)	Zoogonidae							53.7	2	3.7
		Lecithochirium musculus (Looss, 1907) Nasir and Diaz, 1971	Hemiuridae							1.8	0.04	2
		Lepocreadium pegorchis (Stossich, 1901)	Lepocreadiidae	14.3	0.1	1				1.8	0.2	1
		Macvicaria bartolii Antar, Georgieva, Gargouri and Kostadinova, 2015	Opecoelidae	14.3	0.4	3						
		Macvicaria crassigula (Linton, 1910)	Opecoelidae				11.60	0.13	1.2	29.6	1	3.7
		Monorchis parvus Looss, 1902	Monorchiidae	14.3	4.6	32				72.2	11.7	16.2
		Pseudopycnadena fischthali Saad-Fares and Maillard, 1986	Opecoelidae							3.7	0.1	2.5
Diplodus sargus sargus (Linnaeus, 1758) (n=59)	Sparidae	*Arnola microcirrus* (Vlasenko, 1931)	Derogenidae	1.7	0.02	1						
		Diphterostomum brusinae (Stossich, 1889)	Zoogonidae	13.6	0.5	3.7				31.9	7.2	22.5
		Holorchis pycnoporus Stossich, 1901	Aephnidiogenidae	6.8	0.1	1.5				20.3	1.1	5.4
		Lepidauchen stenostoma Nicoll, 1913	Acanthocolpidae							1.5	0.01	1
		Lepocreadium album (Stossich, 1890)	Lepocreadiidae	3.4	0.07	2				2.9	0.06	2
		Macvicaria crassigula (Linton, 1910)	Opecoelidae	23.7	0.3	1.4				24.6	0.7	2.8
		Monorchis parvus Looss, 1902	Monorchiidae							20.2	3.7	15.2
		Proctoeces maculatus (Looss, 1901)	Fellodistomidae	8.5	0.1	1.6				7.2	0.2	2.6
		Pseudopycnadena fischthali Saad-Fares and Maillard, 1986	Opecoelidae	6.8	0.07	1				4.3	0.1	1.3
		Wardula sarguicola Bartoli and Gibson, 1989	Mesometridae	6.8	0.1	1.5				6	0.1	1.5
		Zoogonus rubellus (Olsson, 1868)	Zoogonidae	5.08	0.08	1.7				13	2.6	20.2
Diplodus vulgaris (Geoffroy Saint-Hilaire, 1817) (n=6)	Sparidae	*Diphterostomum brusinae* (Stossich, 1889)	Zoogonidae	66.7	2.3	3.5	6.06	0.33	5.5	34.9	9.2	26.3
		Holorchis pycnoporus Stossich, 1901	Aephnidiogenidae							16.3	1.3	8
		Lepocreadium album (Stossich, 1890)	Lepocreadiidae							2.3	0.07	3
		Macvicaria crassigula (Linton, 1910) Bartoli, Bray and Gibson, 1989	Opecoelidae	16.7	0.2	1	15.15	0.24	1.6	27.9	1.16	4.2
		Monorchis parvus Looss, 1902	Monorchiidae	66.7	4.5	6.7				11.6	1.6	13.8
		Proctoeces maculatus (Looss, 1901)	Fellodistomidae							2.3	0.02	1
		Pseudopycnadena fischthali Saad-Fares and Maillard, 1986	Opecoelidae				6.06	0.04	1.0	13.9	0.1	0.8
		Pycnadenoides senegalensis Fischthal and Thomas, 1972	Opecoelidae							2.3	0.02	1
		Wardula sarguicola Bartoli and Gibson, 1989	Mesometridae							2.3	0.02	1
		Zoogonus rubellus (Olsson, 1868)	Zoogonidae	16.7	0.2	1				7	0.8	11.7

Locality				Bay of Bizerte			Gulf of Gabes			Scandola Nature Reserve		
References				Present study			Derbel *et al.* (2012)			Bartoli *et al.* (2005)		
Fish species (number of specimens)	Family	Digenean species	Family	P(%)	A	MI	P(%)	A	MI	P(%)	A	MI
Lithognathus mormyrus (Linnaeus, 1758) (n=12)	Sparidae	*Derogenes latus* Janiszewska, 1953	Derogenidae							1.9	0.02	1
		Diphterostomum brusinae (Stossich, 1889)	Zoogonidae							3.8	0.06	1.5
		Holorchis pycnoporus Stossich, 1901	Aephnidiogenidae	16.7	6.08	36.5	20	0.56	2.83	50	2,1	4.2
		Lepocreadium pegorchis (Stossich, 1901)	Lepocreadiidae	25	0.9	3.7				5.8	0.7	12.7
		Macvicaria mormyri (Stossich, 1885)	Opecoelidae	8.3	0.2	2	10	0.2	2.0	25	0.4	1.5
		Pycnadenoides senegalensis Fischthal and Thomas, 1972	Opecoelidae							3.8	0.15	4
Oblada melanura (Linnaeus, 1758) (n=79)	Sparidae	*Hemiurus communis* Odhner, 1905	Hemiuridae							4.5	0.05	1
		Lepocreadium album (Stossich, 1890)	Lepocreadiidae	12.7	0.3	2.5				31.8	6	19
		Macvicaria dubia (Stossich, 1905)	Opecoelidae	8.9	0.10	1.4				31.8	0.7	2.1
		Magnibursatus bartolii Kostadinova, Power, Fernández, Balbuena, Raga and Gibson, 2003	Derogenidae	1.3	0.01	1						
Pagellus erythrinus (Linnaeus, 1758) (n=100)	Sparidae	*Allopodocotyle jaffensis* (Fischthal, 1980)	Opecoelidae							2.2	0.02	1
		Hemiurus communis Odhner, 1905	Hemiuridae							3.3	0.15	4.7
		Hemiurus luehei Odhner, 1905	Hemiuridae							2.2	0.1	3
		Holorchis micracanthum (Stossich, 1888)	Aephnidiogenidae	10	0.2	2.1				10.9	0.3	2.3
		Holorchis pycnoporus Stossich, 1901	Aephnidiogenidae	19	0.3	1.7				19.6	0.8	4
		Lepidauchen stenostoma Nicoll, 1913	Acanthocolpidae							1.1	0.01	1
		Lepocreadium album (Stossich, 1890)	Lepocreadiidae	3	0.06	2				4.3	0.04	2.8
		Lepocreadium pegorchis (Stossich, 1901)	Lepocreadiidae	2	0.02	1						
		Macvicaria crassigula (Linton, 1910)	Opecoelidae	5	0.06	1.2				9.8	0.2	1.7
		Pachycreadium carnosum (Rudolphi, 1819) Cortini and Ferretti, 1959	Opecoelidae	8	0.1	1.2				28.3	0.5	1.7
		Pycnadenoides senegalensis Fischthal and Thomas, 1972	Opecoelidae	1	0.01	1						
Pagrus pagrus (Linnaeus, 1758) (n=31)	Sparidae	*Allopodocotyle jaffensis* (Fischthal, 1980)	Opecoelidae							16.9	0.3	1.6
		Aphallus rubalo (Bray, 1986)	Cryptogonimidae	3.2	0.1	3				1.7	0.05	3
		Hemiurus communis Odhner, 1905	Hemiuridae							5.1	0.1	2.3
		Hemiurus luehei Odhner, 1905	Hemiuridae							1.7	0.07	4
		Holorchis micracanthum (Stossich, 1888)	Aephnidiogenidae Lepocreadiidae	6.4	0.06	1				1.7	0.02	1
		Holorchis pycnoporus Stossich, 1901	Aephnidiogenidae	12.9	0.16	1.2				15.3	0.4	2.7
		Lecithochirium musculus (Looss, 1907)	Hemiuridae							3.4	0.03	1
		Lepocreadium album (Stossich, 1890)	Lepocreadiidae									
		Lepocreadium pegorchis (Stossich, 1901)	Lepocreadiidae									
		Macvicaria crassigula (Linton, 1910)	Opecoelidae	9.7	0.1	1.3				27.1	1	3.6
		Pachycreadium carnosum (Rudolphi, 1819)	Opecoelidae							25.4	0.5	2.1
		Zoogonus rubellus (Olsson, 1868)	Zoogonidae							3.4	0.05	1.5

Locality				Bay of Bizerte			Gulf of Gabes			Scandola Nature Reserve		
References				**Present study**			**Derbel *et al.* (2012)**			**Bartoli *et al.* (2005)**		
Fish species (number of specimens)	**Family**	**Digenean species**	**Family**	**P(%)**	**A**	**MI**	**P(%)**	**A**	**MI**	**P(%)**	**A**	**MI**
Sarpa salpa (Linnaeus, 1758) (n=17)	Sparidae	*Centroderma spinosissima* (Stossich, 1883)	Mesometridae	17.6	1.5	8.3	10	0.95	9.5	44.4	13.6	30.5
		Elstia stossichianum (Monticelli, 1892)	Mesometridae							11.1	3.6	32
		Lepocreadium pegorchis (Stossich, 1901)	Lepocreadiidae	5.9	0.2	4	30	3.05	10.16	22.2	0.4	2
		Mesometra brachycoelia Lühe, 1901	Mesometridae	23.5	1.5	6.2	35	0.5	1.4	55.6	17.7	31.8
		Mesometra orbicularis (Rudolphi, 1819)	Mesometridae	11.8	0.5	4.5	60	4.2	7	77.8	11.2	14.4
		Robphildollfusium fractum (Rudolphi, 1819)	Gyliauchenidae	41.2	2.2	5.4	50	6.55	13.10	77.8	34.3	44.1
		Wardula capitellata (Rudolphi, 1819)	Mesometridae	17.6	0.2	1	10	0.1	1	44.4	1.2	2.8
Sparus aurata Linnaeus, 1758 (n=39)	Sparidae	*Allopodocotyle pedicellata* (Stossich, 1887)	Opecoelidae	5.1	0.08	1.5	9.09	0.13	1.5	57.9	3.3	5.7
		Diphterostomum brusinae (Stossich, 1889)	Zoogonidae	5.1	0.05	1				26.3	6.7	25.6
		Lepocreadium pegorchis (Stossich, 1901)	Lepocreadiidae							52.7	1.5	2.9
		Macvicaria maillardi Bartoli, Bray and Gibson, 1989	Opecoelidae	5.1	0.1	2.5				52.7	1.5	2.9
		Macvicaria obovata (Molin, 1859)	Opecoelidae	5.1	0.1	2	36.36	0.9	2.5	52.7	3	5.8
		Magnibursatus bartolii Kostadinova, Power, Fernández, Balbuena, Raga and Gibson, 2003	Derogenidae	2.6	0.03	1						
		Pycnadenoides senegalensis Fischthal and Thomas, 1972	Opecoelidae	2.6	0.05	2				26.3	2.3	8.8
		Zoogonus rubellus (Olsson, 1868)	Zoogonidae							10.5	1	9.5
Spondyliosoma cantharus (Linnaeus, 1758) (n=7)	Sparidae	*Arnola microcirrus* (Vlasenko, 1931)	Derogenidae							0.9	0.01	1
		Hemiurus communis Odhner, 1905	Hemiuridae							2.8	0.1	3.3
		Lepocreadium album (Stossich, 1890)	Lepocreadiidae	14.3	0.3	2				2.8	0.1	3
		Macvicaria bartolii Antar, Georgieva, Gargouri and Kostadinova, 2015	Opecoelidae	57.1	0.6	1						
		Macvicaria crassigula (Linton, 1910)	Opecoelidae							2.8	0.03	1
		Monorchis monorchis (Stossich, 1890)	Monorchiidae							7.4	0.4	4.8
		Steringotrema pagelli (van Beneden, 1871)	Fellodistomidae	28.6	1.3	4.5						

1948 and *Robphildollfusium martinezgomezi* López-Román, Gijon-Botella, Kim and Vilca-Choque, 1992) are considered rare (prevalence < 10 %). Some species (e.g. *Aphanurus stossichii*, *Bacciger israelensis* Fischthal, 1980, *Monascus filiformis* (Rudolphi, 1819), *Opecoeloides furcatus* (Bremser in Rudolphi, 1819) and *Proctotrema bacilliovatum* Odhner, 1911) exhibit a higher level prevalence (10 – 50 %) and are considered as intermediate species. Some others can be rare, intermediate or common (prevalence > 50 %), depending on the host species; for example, this is the case for *Diphterostomum brusinae* (Stossich, 1889), *Ectenurus lepidus* Looss, 1907, *Holorchis micracanthum* (Stossich, 1888), *H. pycnoporus*, *Lepocreadium pegorchis* (Stossich, 1901) and *Macvicaria bartolii*. Such a variation is undoubtedly related to the presence of preferred host species.

The majority of digeneans in the Bay of Bizerte (26 species = 66.7 %) parasitize a single host species, eight were found in two host species and three were collected from four host species. *D. brusinae* and *Lepocreadium album* (Stossich, 1890) were recovered from three and five host species, respectively. The mean number of hosts per species is 1.61. In the light of these results, the overwhelming pattern of host-specificity in the Bay of Bizerte seems to be high. However, despite the availability of the other potential hosts, some species, such as *Aphallus rubalo* (Bray, 1986), *Aphanurus stossichii*, *Hemiurus communis* Odhner, 1905, *Pachycreadium carnosum* (Rudolphi, 1819), *Pseudopycnadena fischthali* Saad-Fares and Maillard, 1986 and *Wardula sarguicola* Bartoli and Gibson, 1989, infect a single host species in the Bay of Bizerte, whereas they are known to have no strict host-specificity in the Mediterranean region (Sasal *et al*., 1999; Bartoli *et al*., 2005). Previous studies have shown that the variety in the diet of a host species, its vagility, its relatedness to other sympatric host species and how long it has been in the area can influence the chances of a host acquiring parasite species (Price & Clancy, 1983; Kennedy *et al*., 1986; Kennedy & Bush, 1994). It is notable that *E. lepidus*, a common parasite of a wide range of marine teleosts (Atherinidae, Carangidae, Sparidae, Lophiidae, Scombridae) was recovered from only two hosts, *B. boops* and *T. trachurus*, in the Bay of Bizerte; the former is a new host record for this digenean. We admit that further sampling of a broad range of fish families may well change our understanding of the patterns of host-specificity in the Bay of Bizerte.

Discussion

The mean number of 2.58 species per host, estimated from published data on digenean parasites of labrid fish from the Bay of Bizerte (Gargouri *et al*., 2010) and the present study, is distinctly lower than that reported for teleost hosts in the Scandola Nature Reserve off Corsica (3.8, see Bartoli *et al*., 2005) and significantly higher than that observed for teleosts off the southern coast of Tunisia (1.7, see Derbel *et al*., 2012). Bartoli and co-workers (2005) reported for different basins of the Mediterranean a range of mean values (0.6 – 2.9 species per host). The comparison of our data and these reports shows similar digenean species richness to that in teleosts from Eastern Mediterranean and a greater digenean diversity than that reported in teleosts from the Adriatic and north-western Italian coast (see Bartoli *et al*., 2005). Furthermore, in a recent study of the trematode fauna of the Mediterranean, Pérez-del-Olmo *et al*. (2016) estimated a lower overall mean number (1.57 species per host) indicating, according to these authors, a high diversity in the Mediterranean. However, these authors stated that they did not consider geographical variation in terms of faunal richness and that their estimate was rather artificial. Similar results were obtained when we, taking into account the work carried out by Gargouri *et al*. (2010), compared the data reported for the same 10 teleost species studied in the Bay of Bizerte, the Gulf of Gabes and the Scandola Nature Reserve. An intermediate situation for the Bay of Bizerte was encountered, with a mean number of 3.8 species per host, which is much higher than that for the southern coast of Tunisia (2.1 species per host) and lower than that for the Scandola Nature Reserve (4.2 species per host). The environment at the Bay of Bizerte appears to provide favourable conditions for the transmission of digeneans and the completion of their life-cycles. Among the possible reasons explaining the relatively high digenean diversity in teleosts from the Bay of Bizerte is its situation in the eastern part of the Western Mediterranean (FAO division 37.1.3 Sardinia) and its connection with the neighbouring Mediterranean lagoon, i.e. Bizerte Lagoon, via a channel. Indeed, several studies (Bartoli, 1974; Maillard, 1976; Thomas *et al*., 1997; Bartoli & Gibson, 2007) have pointed out the importance of parasitism in lagoonal ecosystems; these shallow and confined biotopes with high specific diversity and high predation levels favour the life-cycles and the transmission of parasites. Moreover, Saad Fares (1985) attributed the low digenean diversity observed off the Lebanese coasts to obstacles to the completion of the life-cycle of parasites on these coasts, which are characterized by the absence of lagoons and ponds. On the other hand, the north coast of Tunisia presents a varied topography. In fact, the substrata are mainly rocky but also both hard and soft; this variety of biotopes enables a high biodiversity (Boudouresque, 1997). The mainly rocky bottoms of the littoral marine areas of the northern Tunisian coasts offer the best substrata for the colonization of very rich, coralligenous assemblages (Ben Mustapha *et al*., 2002). *Posidonia oceanica* meadows are also well represented in this part of the Tunisian coast and are geographically extensive off Bizerte and Cani (Ben Mustapha *et al*., 2002). Both biocoenoses exhibit a high diversity of macro-benthic organisms (Ben Mustapha *et al*., 2002), some of which act as intermediate hosts for several digeneans species. In addition, *Posidonia oceanica* meadows represent productive ecosystems that provide habitats and food resources for a diverse fish fauna and act as important nursery areas for many coastal species (Harmelin-Vivien, 1982). Also, due to the nature of the sea bottom off the northern region of Tunisia, some fishing methods, such as bottom trawling, are not practiced. Bottom trawling is considered one of the most disruptive human-induced physical disturbances to seabed communities (Rumohr & Krost, 1991). Indeed, previous studies have shown that the biomass and abundance of benthic organisms is reduced by trawling, leading to long-term changes in the benthic species composition (Reise, 1982; Reise & Schubert, 1987; Thrush *et al*., 1991). Engel & Kvitek (1998) provided evidence that high levels of trawling can decrease bottom habitat complexity and biodiversity. Derbel *et al*. (2012) attributed the low digenean richness in the Gulf of Gabes to the disturbance resulting from changes in the structure and the function of the marine ecosystem off southern Tunisia caused by human activities (overfishing and the use of the trawling) and the impact of exotic species (the introduction of invasive algae). Conversely, the high digenean diversity reported for the Scandola Nature Reserve is related to its high general level of biodiversity (Miniconi *et al*., 1990; Verlaque, 1990; Verlaque *et al*., 1999) and related to the stability of the equilibrium of the ecosystem, which has been strictly protected since 1979 and where spear-fishing and angling are prohibited.

Examination of the level of infection revealed that digeneans, in general, exhibited a higher prevalence in the Scandola Nature Reserve and Gulf of Gabes than in the Bay of Bizerte. There are many reasons which might explain the origin of this differential distribution of digenean frequencies between the different Mediterranean environments. The high level of prevalence reported for the Gulf of Gabes appears to be related to a greater sampling effort (534 teleosts of 14 species for the Bay of Bizerte *vs* 779 teleosts of 32 species for the Gulf of Gabes) and to the abundance of intermediate hosts, and in particular the molluscan host, in this locality. Furthermore, during their free-living phases, parasites of aquatic organisms with complex life-cycles are negatively affected by changes of environmental conditions, including urban and industrial pollution and other anthropogenic perturbations (Pietrock & Marcogliese, 2003). They are sensitive to environmental stressors such as wastewater or industrial pollutants and chemicals released into the environment as a result of human activities and their transmission is impeded or modified in polluted habitats (MacKenzie *et al*., 1995; Marcogliese & Cone, 1997). These pollutants are toxic and lead to a decrease in the prevalence and abundance of parasites by acting directly on the parasites themselves and their free-living stages, or indirectly on density of the definitive or intermediate hosts (Overstreet & Howse, 1977; Khan & Thulin, 1991; Poulin, 1992; MacKenzie *et al*., 1995; Lafferty, 1997; Sures, 2004, 2008). According to MacKenzie *et al*. (1995), pollutants may influence the infection levels of endohelminths in fish hosts by affecting the numbers of parasite larval stages carried by invertebrate intermediate hosts or by causing changes in the pathogenicity of invertebrate pathogens. In the Bay of Bizerte, the major source of pollution is a large oil refinery. This refinery has, since 1999, imported all of Tunisia's requirements for petroleum products, and its potential as a direct source of oil pollution has been accentuated by an increasing production of refined products. Indeed, a simple direct observation has revealed the non-negligible presence of hydrocarbons in the wastewater of this refinery released into the marine environment (Boufahja, 2010). Moreover, oil traffic can also, even accidentally, result in the discharge of crude or refined petroleum products during load shedding or pumping operations (Beyrem, 1999). Some laboratory experiments and studies of wild-caught fish suggest that exposure to oil and its components reduces the prevalence and intensity of gastrointestinal helminths (Haensly *et al*., 1982; Kiceniuk & Khan, 1983). Furthermore, Khan and Kiceniuk (1983) have suggested that the low species diversity and the low infection intensity of gut parasites in fish exposed to oil might be attributed to its toxicity acting directly on the parasites and/or causing a modification of the gut environment which becomes inhospitable to the parasites; the latter is brought about by changes in fish physiology. On the other hand, the invasive seaweed *Caulerpa taxifolia*, reported for the first time in Tunisian waters by Langar *et al*. (2000), synthesizes toxic metabolites which are released into the environment. These metabolites can affect the behaviour, survival and transmission of infective, free-living, larval stages to the intermediate hosts (Bartoli & Boudouresque, 1997). Digenean free-living stages are also sensitive to the action of exogenous environmental factors, such as temperature, salinity, hydrogen ion concentration, water level and photoperiod. The variation of these abiotic factors influences the prevalence of parasites and their presence in both time and space (Chubb, 1979, 1980) by affecting the rates of cercarial emergence and the transmission success.

In conclusion, our comparative study showed a high diversity of digenean species in teleost fishes off the northern coast of Tunisia suggesting that the Bay of Bizerte provide favourable conditions for the transmission of these helminths and the completion of their life-cycles.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Comparison of the endoparasite fauna of *Hoplias malabaricus* and *Hoplerythrinus unitaeniatus* (Erythrinidae), sympatric hosts in the eastern Amazon region (Brazil)

M. S. B. OLIVEIRA[1,5]*, L. LIMA CORRÊA[2], L. PRESTES[3], L. R. NEVES[4,5], A. R. P. BRASILIENSE[1,5], D. O. FERREIRA[5], M. TAVARES-DIAS[1,4,5]

[1]Postgraduate Program in Tropical Biodiversity - PPGBIO, Universidade Federal do Amapá - UNIFAP, Rod. Juscelino Kubitschek, KM-02, CEP 68.903-419, Jardim Marco Zero, Macapá, Amapá, Brazil, *E-mail: *marcosidney2012@hotmail.com*; [2]Universidade Federal do Oeste do Pará - UFOPA, Av. Mendonça Furtado, nº 2946, Fátima, CEP 68040-470, Instituto de Ciências e Tecnologia das Águas - ICTA, Santarém, Pará, Brazil, E-mail: *lincorre@gmail.com*; [3]Postgraduate Program in Aquatic Ecology and Fisheries - PPGEAP, Universidade Federal Rural da Amazônia - UFRA. Av. Presidente Tancredo Neves, nº 2501, Terra Firme, CEP 66077-830, Belém, Pará, Brazil and Universidade do Estado do Amapá - UEAP, Av. Presidente Vargas nº 100, CEP 66077-830, Central, Macapá, Amapá, Brazil, E-mail: *luliprestes@gmail.com*; [4]Postgraduate Program in the Biodiversity and Biotechnology of the Legal Amazon - PPGBIONORTE - AP. Universidade Federal do Amapá - UNIFAP, Rod. Juscelino Kubitschek, KM-02, CEP 68.903-419, Jardim Marco Zero, Macapá, Amapá, Brazil. E-mail: *liahrigor@hotmail.com*; [5]Embrapa Amapá, Rodovia Juscelino Kubitschek, Km 5, nº 2600, Universidade, CEP 68903-419, Macapá, Amapá, Brazil, E-mail: *driellyferreira2015@outlook.com*; *marcos.tavares@embrapa.br*

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Summary

Hoplias malabaricus and *Hoplerythrinus unitaeniatus* are Erythrinidae family widely distributed in the Amazon River system of great value to both commercial and subsistence fishing for riverine populations. As such, the objective of the present study was to investigate the endoparasite communities of *H. malabaricus* and *H. unitaeniatus* of a tributary of the Amazon River in the north of Brazil. The endoparasite communities of *H. unitaeniatus* and *H. malabaricus* were taxonomically similar (85%) and consisted of *Clinostomum marginatum, Contracaecum* sp., *Guyanema seriei seriei*, *Procamallanus* (*Spirocamallanus*) *inopinatus*, *Pseudoproleptus* sp. and *Gorytocephalus spectabilis*, although the dominant endoparasite was *C. marginatum*, which was the most prevalent and abundant. All the specimens of both *H. malabaricus* and *H. unitaeniatus* were parasitized, with a total of 1237 helminths collected in the former host and 1151 helminths collected in the latter. *Hoplerythrinus unitaeniatus* possessed greater parasite species richness. Both hosts had an aggregate dispersion of parasites, and the abundance of *C. marginatum, Contracaecum* sp. and *G. spectabilis* correlated positively with the weight and length of the hosts. The condition factor was not affected by parasitism, but the abundance of *C. marginatum* and *Contracaecum* sp. increased when the condition factor of the hosts decreased. This is the first report of *G. seriei seriei* for *H. malabaricus* and *Pseudoproleptus* sp. for *H. unitaeniatus*.
Keywords: Amazon; Vila Nova River; Erythrinidae; helminth parasites

Introduction

The state of Amapá has 34 hydrographic basins, including the Vila Nova River basin, which is one of the largest in the state (Zee, 1997; Silva *et al*., 2006), and covers the municipalities of Santana and Mazagão, flowing into the Amazon River near Santana. The Vila Nova is a white-water river with a pH of 5-7 (Cunha, 2003). Besides being important for navigation and water supply (Silva *et al*., 2006), it is home to several species of fish, including the Erythrinidae family (including *Hoplias malabaricus* Bloch, 1794 and *Hop-*

* – corresponding author

lerythrinus unitaeniatus Spix & Agassiz, 1829), as it possesses an extensive flood plain area. Amazon floodplain lakes are complex environments, whose spatial heterogeneity spans such distinct habitats as flooded forests, macrophyte meadows and open water. These habitats provide areas which are used by several fish species for shelter, feeding, growth and reproduction during different phases of their life cycles. The seasonal component (rainy and dry seasons) adds additional complexity to the floodplain habitat by altering the availability of these habitats to fish over the course of the year (Siqueira-Souza *et al*., 2017).

Hoplias malabaricus and *H. unitaeniatus* are common Erythrinidae from the Amazon River system, and are important for commercial and subsistence fishing (Santos *et al*., 2006; Soares *et al*., 2011; Gonçalves *et al*., 2016). Both fish inhabit rivers, lakes and flooded forests (Mattox *et al*., 2006). The young of *H. malabaricus* and *H. unitaeniatus* feed on plankton such as microcrustaceans and insects, while adults feed mainly on fish and shrimp. They can tolerate low concentrations of dissolved oxygen in the water and take care of their offspring (Santos *et al*., 2006; Soares *et al*., 2011). The occurrence of *H. malabaricus* and *H. unitaeniatus* can be observed in various environments, and their carnivorous diet and elevated position in the food chain, makes them good host models in parasitic ecology (Alcântara & Tavares-Dias, 2015; Gonçalves *et al*., 2016).

In the Amazon region of South America, the parasite fauna of *H. malabaricus* and *H. unitaeniatus* consists of 17 species, of which seven are endoparasites of *H. unitaeniatus* and ten are endoparasites of *H. malabaricus* (Alcântara & Tavares-Dias, 2015; Gonçalves *et al*., 2016). The rainy and dry seasons create variations in the availability of food in Amazon habitats, leading to fluctuations in the infracommunities of the parasites of the two hosts (Gonçalves *et al*., 2016). However, the size of these two hosts has no relation to the abundance of parasites (Alcântara & Tavares-Dias, 2015; Gonçalves *et al*., 2016).

The relationship between parasites and hosts can be regulated by the host mortality induced by parasites. Abundant hosts generally tend to harbor richer parasite fauna, but if the host species are less numerous, their parasite fauna may become less rich (Morozińska-Gogol, 2015). As *H. malabaricus* and *H. unitaeniatus* are abundant hosts in the floodplain area of the Amazon River system and have a similar life history, will they present a similar community of endoparasites? Populations of hosts with a similar life history that live in the same geographical area and are exposed to the same infection stages may present a qualitatively and quantitatively similar community of endoparasites when they ingest similar quantities and types of prey. In this manner, overlapping in the same area of occurrence may have an important effect on endoparasite communities in phylogenetically related hosts (Alarcos & Timi, 2012; Alcântara & Tavares-Dias, 2015; Hoshino *et al*., 2016; Oliveira *et al*., 2016).

Parasites can regulate the growth of host fish populations, reducing fertility and affecting the swimming, feeding and behavior of these animals (Corrêa *et al*., 2014; Machado *et al*., 2013; Hoshino *et al*., 2016). Knowledge of the parasites of natural populations can be important for decision-making regarding the monitoring of fish stocks, as they generate information about the physical conditions of fish (Corrêa *et al*., 2013). As such, the objective of this study was to comparate the endoparasites fauna of *H. malabaricus* and *H. unitaeniatus* in a floodplain area of the basin of the Vila Nova River, a tributary of the Amazon River, Northern Brazil.

Materials and Methods

Fish and collection location

In October 2015 (dry season), 30 specimens of *H. unitaeniatus* and 30 specimens of *H. malabaricus* were captured in the floodplain region of the Vila Nova River in the municipality of Mazagão, a tributary of the Amazon River, in the state of Amapá, Brazil (Fig. 1), for parasitological analysis. Gill nets were used to capture the fish (30 and 35 mm between knots). The fish were transported in boxes with ice to the Laboratory of Aquaculture and Fishery from Embrapa Amapá.

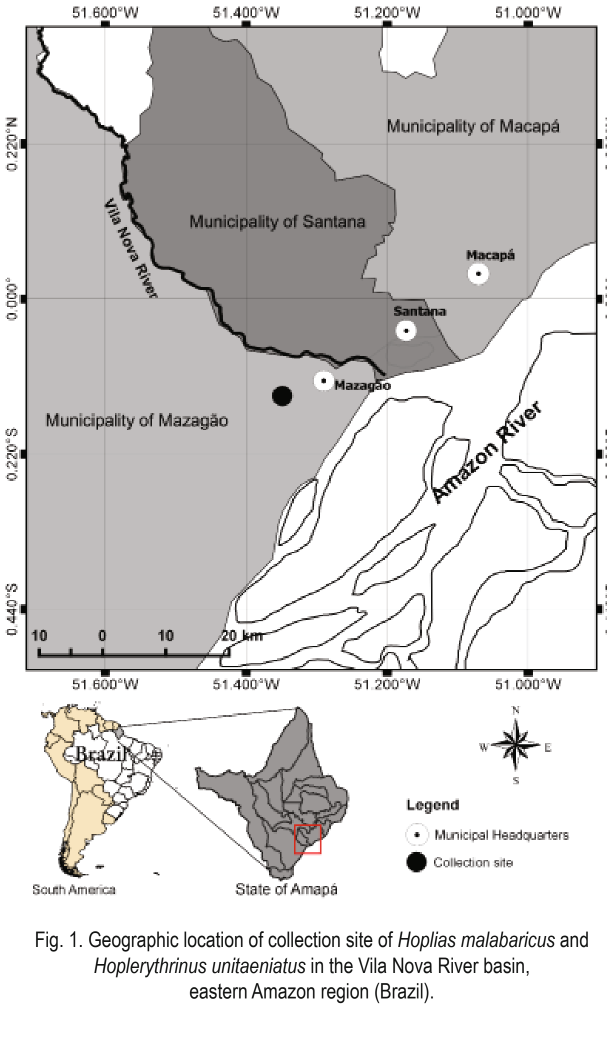

Fig. 1. Geographic location of collection site of *Hoplias malabaricus* and *Hoplerythrinus unitaeniatus* in the Vila Nova River basin, eastern Amazon region (Brazil).

The Vila Nova River and its floodplain areas are strongly influenced by tides through the Amazon River, and in the rainy season fish enter the floodplain areas in search of food. In dry seasons, however, these areas are reduced and have a low dissolved oxygen level (Silva *et al*., 2006).

Collection, fixation and identification procedures of parasites

After collection, the fish were euthanized by the spinal cord transection method and weighed (g) and measured for standard length (cm). The fish were then necropsied for parasitological analysis. This work was carried out in accordance with the principles adopted by the Colégio Brasileiro de Experimento Animal (Brazilian College of Animal Experimentation -Cobea) with the authorization from Ethics Committee in the Use of Animals of Embrapa Amapá (#:004 - CEUA/CPAFAP).

After necropsy, the gastrointestinal tract and viscera were analyzed using a stereomicroscope and a light microscope to collect endoparasites. The methodology used to fix, preserve, quantify and stain the parasites for identification was that recommended by Eiras *et al*. (2006). The parasites were identified in accordance with Petter (1975), Moravec (1998), Moravec & Santos (2009), Vicente & Pinto (1999), Thatcher (2006) and Caffara *et al*. (2011). The ecological terms proposed by Rohde *et al*. (1995) and Bush *et al*. (1997) were used.

Data analysis

The Brillouin diversity index (*HB*), uniformity (*E*), Berger-Parker dominance index (*d*) and species richness of the parasites (Magurran, 2004) was calculated to evaluate the endoparasite component community using the Diversity software package (Pisces Conservation Ltd, UK). The index of dispersion (ID) and discrepancy index (D) were calculated using Quantitative Parasitology 3.0 software to detect the distribution pattern of the parasite infracommunities (Rózsa *et al*., 2000) for species with a prevalence of > 10 %. The significance of the (ID), for each infracommunity, was tested using the *d*-statistics test (Ludwig & Reynolds, 1988). The Jaccard index (J) and Bray-Curtis index (B) were used to measure similarity in parasite abundance between *H. unitaeniatus* and *H. malabaricus*. These take into account the differences in abundance between the shared parasite species (Ludwig & Reynolds, 1988; Magurran, 2004). These similarity indices were calculated using the Past software (Hammer *et al*., 2001). Principal component analysis (PCA) was carried out to compare the ways in which body size and diversity influenced the parasite communities of *H. unitaeniatus* and *H. malabaricus*. This analysis was performed using the Past software (Hammer *et al*., 2001).

The total weight (g) and standard length (cm) of the fish were used to calculate the relative condition factor (Kn) of the hosts and the weight-length ratio using the equation $W = a.L^b$, where W is the total weight (g) and L is the standard length (cm), a and b are con-

Table 1. Helminth parasites of two Erythrinidae species from the Vila Nova River basin, eastern Amazon region (Brazil).
P: Prevalence, MI: Mean intensity, MA: Mean abundance, TNP: Total number of parasites, SI: Site of infection, FD: Frequency of dominance.

Fish species	***Hoplerythrinus unitaeniatus* (N = 30)**						***Hoplias malabaricus* (N = 30)**					
Parasites	**P (%)**	**MI**	**MA**	**TNP**	**FD (%)**	**SI**	**P (%)**	**MI**	**MA**	**TNP**	**FD (%)**	**SI**
Clinostomum marginatum (larvae)	76.7	29.6	21.7	651	0.566	Mesentery	83.3	25.8	21.5	645	0.521	Mesentery
Clinostomum marginatum (larvae)	-	-	-	-	-	-	3.3	2.0	0.07	2	0.002	Intestine
Clinostomum marginatum (larvae)	6.7	1.5	0.10	3	0.002	Musculature	-	-	-	-	-	-
Pseudoproleptus sp. (larvae)	33.3	4.9	1.63	49	0.042	Intestine	96.7	12.9	12.9	388	0.314	Mesentery
Pseudoproleptus sp. (larvae)	26.7	6.8	1.80	54	0.046	Liver	13.3	4.0	0.5	16	0.013	Intestine
Pseudoproleptus sp. (larvae)	13.3	1.8	0.23	7	0.006	Caecum	-	-	-	-	-	-
Pseudoproleptus sp. (larvae)	66.7	5.3	3.50	105	0.090	Mesentery	-	-	-	-	-	-
Contracaecum sp. (larvae)	83.3	3.4	2.87	86	0.074	Mesentery	90.0	5.5	4.8	143	0.116	Mesentery
Contracaecum sp. (larvae)	10.0	3.0	0.10	3	0.002	Cecum	3.3	1.0	0.03	1	0.001	Intestine
Contracaecum sp. (larvae)	10.0	1.0	0.10	3	0.002	Liver	-	-	-	-	-	-
Contracaecum sp. (larvae)	3.3	1.0	0.03	1	0.0009	Intestine	-	-	-	-	-	-
Procamallanus (*S.*) *inopinatus*	26.7	1.4	0.37	11	0.009	Caecum	-	-	-	-	-	-
Procamallanus (*S.*) *inopinatus*	63.3	3.8	2.43	73	0.063	Intestine	-	-	-	-	-	-
Guyanema seriei seriei	3.3	2.0	0.07	2	0.001	Mesentery	-	-	-	-	-	-
Gorytocephalus spectabilis	60.0	3.5	2.10	63	0.054	Intestine	23.3	8.4	1.4	35	0.028	Mesentery
Gorytocephalus spectabilis	3.3	4.0	0.13	4	0.003	Liver						
Gorytocephalus spectabilis	30.0	3.6	1.07	32	0.027	Caecum	-	-	-	-	-	-
Gorytocephalus spectabilis	6.67	2.0	0.13	4	0.003	Mesentery	-	-	-	-	-	-

Table 2. Index od dispersion (ID), d-statistic, and discrepancy index (D) for the infracommunities of parasitic helminths of two species of Erythrinidae from the Vila Nova River basin, eastern Amazon region (Brazil).

Hosts fish	***Hoplias malabaricus* (N = 30)**			***Hoplerythrinus unitaeniatus* (N = 30)**		
Parasites	ID	*d*	D	ID	*d*	D
Gorytocephalus spectabilis (intestine)	2.286	3.965	0.720	2.455	4.383	0597
Pseudoproleptus sp. (intestine)	1.975	3.153	0.871	2.063	3.389	0.730
Pseudoproleptus sp. (liver)	-	-	-	2.138	3.676	0.771
Pseudoproleptus sp. (caecum)	-	-	-	1.680	2.322	0.853
Pseudoproleptus sp. (mesentery)	2.236	3.849	0.323	2.811	5.219	0.507
Procamallanus (*S.*) *inopinatus* (intestine)	-	-	-	2.072	3.413	0.551
Procamallanus (*S.*) *inopinatus* (caecum)	-	-	-	1.219	0.859	0.754
Clinostomum marginatum (mesentery)	4.339	8.314	0.435	12.097	18.939	0.735
Contracaecum sp. (mesentery)	2.462	4.400	0.417	2.325	4.063	0.471
Gorytocephalus spectabilis (cecum)	-	-	-	2.670	4.701	0.767

stants, estimated by the linear regression of the transformed equation: W = log a + b x log Cp. (Le-Cren, 1951). The t-test was used to compare the Kn of hosts with the standard value (Kn = 1.00). The Spearman coefficient (*rs*) was used to determine the possible correlations between parasite abundance and host length, body weight and Kn, as well as to correlate host length with species richness and *HB*. The Mann-Whitney (*U*) test was used to compare the mean intensity, mean abundance, species richness, *HB, E* and Berger-Parker dominance of both host species (Zar, 2010).

Ethical Approval and/or Informed Consent

This work was carried out in accordance with the principles adopted by the Brazilian College of Animal Experimentation (Cobea) with the authorization from Ethics Committee in the Use of Animals of Embrapa Amapá (#:004 - CEUA/CPAFAP).

Results

Thirty specimens of *H. unitaeniatus* measuring $\overline{x}$ = 21.5 ± 2.0 cm and $\overline{x}$ = 245.3 ± 65.6 g, and 30 specimens of *H. malabaricus* with $\overline{x}$ = 24.9 ± 7.7 cm and $\overline{x}$ = 242.3 ± 75.0 g were analyzed.

Of the specimens of *H. unitaeniatus* and *H. malabaricus* examined, 100 % were parasitized by one or more species of helminth. It was observed that there was similar dominance of the digenean *Clinostomum marginatum* Rudolphi, 1819, in *H. malabaricus* and

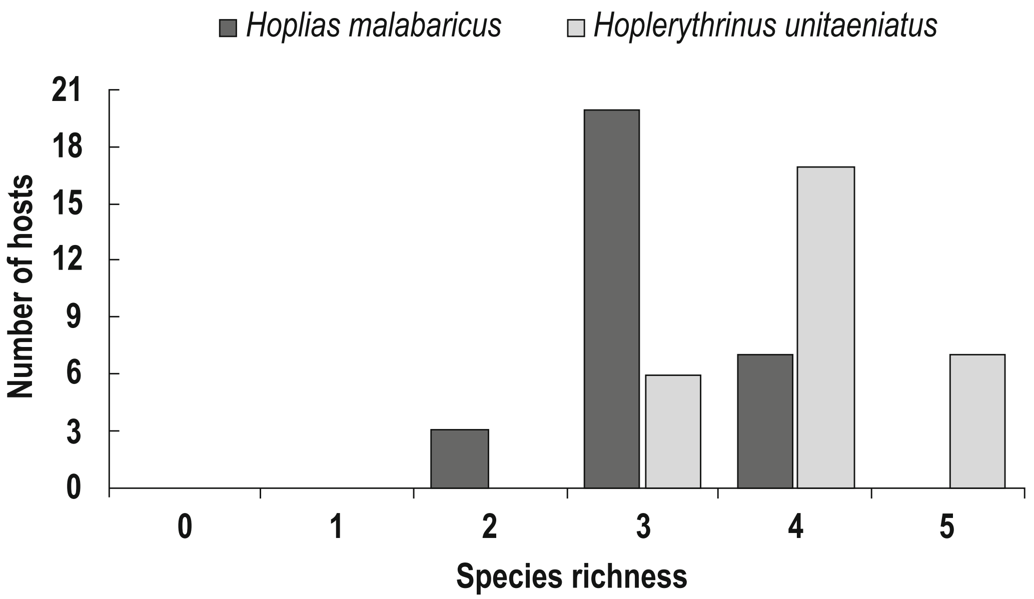

Fig. 2. Species richness of parasitic helminths of parasite helminths of two species of Erythrinidae from the Vila Nova River basin, eastern Amazon region (Brazil).

Table 3. Diversity descriptors for infracommunities of parasitic helminths of two species of Erythrinidae from the Vila Nova River basin, eastern Amazon region (Brazil). *U* = Mann-Whitney.

Mean indices of diversity	***Hoplias malabaricus***	***Hoplerythrinus unitaeniatus***	***U***	***p***
Species richness of parasites	3.1 ± 0.6 (2-4)	4.0 ± 0.6 (3-5)	161.5	**0.0001**
Brillouin (*HB*)	0.72 ± 0.19 (0.24 – 1.0)	0.86 ± 0.19 (0.54 – 1.23)	277.0	**0.0053**
Evenness (*E*)	0.60 ± 0.15 (0.25 – 0.83)	0.57 ± 0.12 (0.32 – 0.77)	378.0	0.1436
Dominance of Berger-Parker (*d*)	0.64 ± 0.13 (0.37 – 0.89)	0.57 ± 0.15 (0.28 – 0.85)	365.5	**0.0467**

H. unitaeniatus, followed by *Contracaecum* sp. for both hosts. A total of 1151 helminths were collected in *H. unitaeniatus* and 1237 in *H. malabaricus*, making a total of 2,388 helminths. These parasites were distributed among the following taxa: *Clinostomum marginatum* (Trematoda), *Guyanema seriei seriei* Petter, 1975, *Procamallanus* (*Spirocamallanus*) *inopinatus* Travassos, Artigas & Pereira, 1928, *Pseudoproleptus* Khera, 1955, *Contracaecum* Railliet & Henry, 1912 (Nematoda) and *Gorytocephalus spectabilis* Machado, 1959 (Acanthocephala) (Table 1). These parasites presented aggregated dispersion, except *P.* (*S.*) *inopinatus* in the pyloric cecum of *H. unitaeniatus* that exhibited a random dispersion (Table 2).

Berger-Parker diversity index and evenness were similar for both fish species, but the Brillouin index (*HB*) and species richness of the parasites were higher for *H. unitaeniatus* (Table 3), and there was no difference between the abundance (U = 430.5, p = 0.309) and parasitic intensity (U = 430.5, p = 0.309) in the two fish species. In *H. malabaricus* there was a predominance of individuals harboring three species of helminths, whereas in *H. unitaeniatus* the predominance was four species of helminths (Fig. 2).

The *H. unitaeniatus* and *H. malabaricus* populations exhibited low parasite community similarity, as described by the Jaccard index (J = 0.66) and the Bray-Curtis index (B = 0.15). Multivariate analysis based on the parasite communities of *H. unitaeniatus* and *H. malabaricus* revealed a difference between these host populations, caused by *C. marginatum* and *Pseudoproleptus* sp. (Fig. 3).

For *H. malabaricus*, the abundance of *C. marginatum* correlated positively with the length and negatively with the Kn of the hosts. In the same manner, the abundance of *Contracaecum* sp. correlated positively with host size and negatively with Kn. For *H. unitaeniatus*, there was a negative correlation between the abundance of *G. spectabilis* and host length, while the abundance of *C. marginatum* correlated positively with host length and body weight. The abundance of *Contracaecum* sp. also exhibited a positive correlation with host length (Table 4).

The condition factor of the parasitized *H. malabaricus* (Kn = 0.999 ± 0.063) did not differ (t = -0.062; p = 0.951) from the standard (Kn = 1.00), and the same was true for *H. unitaeniatus* (Kn = 1.00 ± 0.017) (t = 0.003, p = 0.997). The equation describing the growth of

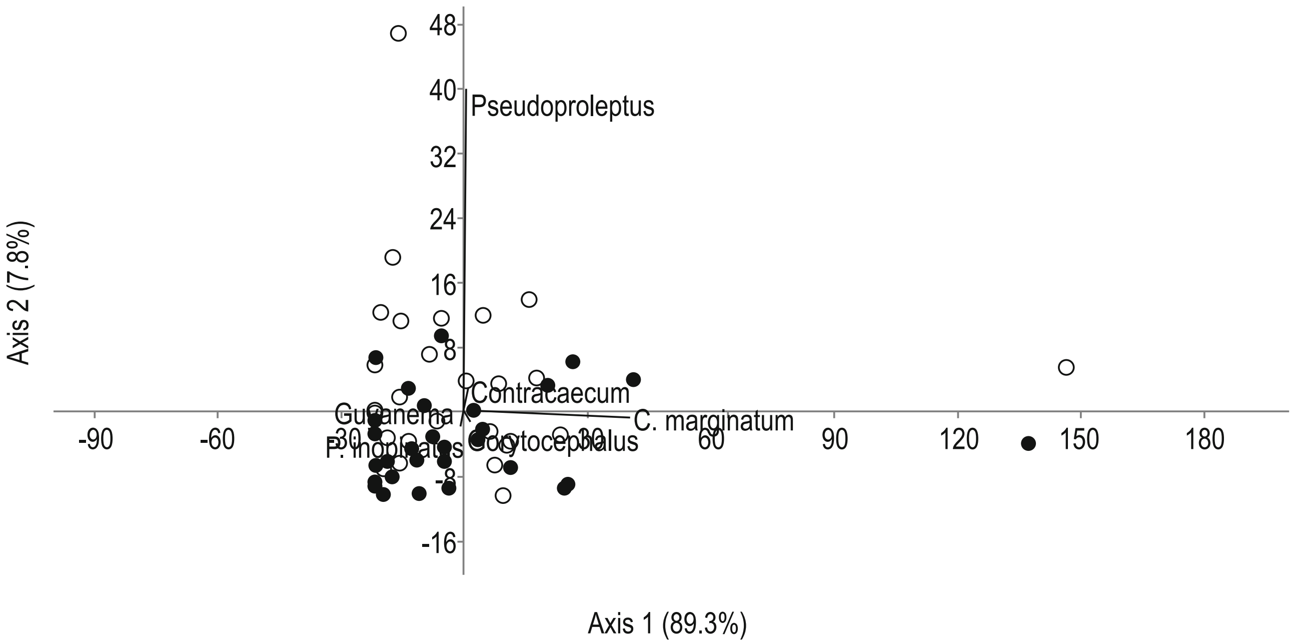

Fig. 3. Scatterplot scores of the principal component analysis (PCA) on endoparasites of de *Hoplias malabaricus* (O) e *Hoplerythrinus unitaeniatus* (●) from Vila Nova River, eastern Amazon (Brazil). P. inopinatus: *Procamallanus* (*Spirocamallanus*) *inopinatus*, Guyanema: *Guyanema serieri serieri*, Gorytocephalus: *Gorytocephalus spectabilis*, Pseudoproleptus: *Pseudoproleptus* sp., C.marginatum: *Clinostomum marginatum*.

Table 4. Spearman correlation coefficient (*rs*) of abundance of parasites with standard length, body weight and Kn for the infracommunities of parasite helminths of two species of Erythrinidae from the Vila Nova River basin, eastern Amazon region (Brazil).

Hosts fish	***Hoplias malabaricus***						***Hoplerythrinus unitaeniatus***					
	Length		Weight		Kn		Length		Weight		Kn	
Parasites	*rs*	p	*rs*	p	*rs*	p	*rs*	p	*rs*	p	*rs*	p
Pseudoproleptus sp.	0.284	0.127	0.293	0.115	-0.239	0.202	0.253	0.177	0.117	0.537	-0.130	0.493
Gorytocephalus spectabilis	-0.159	0.400	-0.252	0.177	0.035	0.851	-0.432	**0.017**	-0.304	0.101	0.041	0.826
Clinostomum marginatum	0.467	**0.001**	0.361	0.049	-0.593	**0.0005**	0.454	**0.011**	0.5671	**0.001**	-0.186	0.322
Contracaecum sp.	0.545	**0.001**	0.539	**0.002**	-0.5137	**0.0037**	0.347	**0.059**	0.2314	0.218	0.033	0.859
Procamallanus (*S.*) *inopinatus*	-	-	-	-	-	-	-0.073	0.700	-0.0008	0.996	0.213	0.257
Guyanema s. seriei	-	-	-	-	-	-	-0.253	0.176	-0.246	0.188	-0.032	0.865

H. malabaricus was $W = 0.0891L^{2.5057}$; $r^2 = 0.898$, while for *H. unitaeniatus* it was $W = 0.0327L^{2.8978}$; $r^2 = 0.904$, which shows negative allometric type growth.

Discussion

The endoparasite fauna in *H. malabaricus* was composed by 1 species of Digenea, 4 Nematoda and 1 Acanthocephala, while in *H. unitaeniatus* it consisted of 1 species of Digenea, 2 Nematoda and 1 Acanthocephala. Thus, 66.6 % of these taxa are known species for these hosts in the eastern Amazon region. The endoparasite communities of *H. unitaeniatus* and *H. malabaricus* were dissimilar (15 %) and were mostly influenced by the amount of ingested prey. However, a certain degree of homogeneity can be expected in hosts living in the same environment that are phylogenetically related and have a similar ecology (Alarcos & Timi, 2012; Hoshino *et al*., 2016). The parasites of *H. unitaeniatus* and *H. malabaricus* presented aggregate dispersion, but *H. unitaeniatus* demonstrated greater species richness, a higher Brillouin index and lower Berger-Parker dominance. The greater species richness of endoparasites of *H. unitaeniatus* is an indication that their feeding is more diversified than *H. malabaricus* in the studied environment. This higher species richness of endoparasites in *H. unitaeniatus* can therefore result in a greater number of infected organs, thus causing a reduction in competition among endoparasites.

The parasite dispersion pattern in both *H. malabaricus* and *H. unitaeniatus* was aggregated, a pattern registered for others freshwater fish in Brazil (Luque *et al*., 2003, Guidelli *et al*., 2003; Tavares-Dias *et al*., 2014a,b; Oliveira *et al*., 2016, 2017). This pattern is mainly influenced by the breadth of the ecological niche dimension, environmental heterogeneity and host immunology (Anderson & Gordon, 1982; Guidelli *et al*., 2003; Tavares-Dias *et al*., 2013; Oliveira *et al*., 2016). However, the infection by *P.* (*S.*) *inopinatus* in the pyloric cecum of *H. unitaeniatus* had a random dispersion, similar to the infection of this nematode in the pyloric cecum of *T. angulatus* from the Amazon River system (Oliveira *et al*., 2016). The random dispersion pattern is common in larvae and species of parasites with a high degree of pathogenicity, and that have a reduced possibility of colonizing hosts (Guidelli *et al*., 2003). Therefore, such parasite dispersion patterns may vary depending on the colonization strategies of the parasite species.

The growth type of *H. malabaricus* and *H. unitaeniatus* was negative allometric, indicating a greater increase in length than in body mass. In both *H. malabaricus* and *H. unitaeniatus*, there was a positive correlation between the abundance of *C. marginatum* and *Contracaecum* sp. and the size of the hosts. This is a strong indicator of the accumulation of these endoparasites throughout the life of these hosts, influenced mainly by the greater possibility of intermediate host ingestion, and a longer time of exposure to parasitic infections (Guidelli *et al*., 2003; Bicudo *et al*., 2005; Bellay *et al*., 2012). However, *H. malabaricus* and *H. unitaeniatus*, which are fish of sedentary habits (Santos *et al*., 2006, Soares *et al*., 2011), exhibited differences in the number of prey containing infective forms of the endoparasites found, thus demonstrating a relative overlap in the same environment investigated. The negative correlation between the abundance of *C. marginatum* and *Contracaecum* sp. and the size of *H. malabaricus* and the condition factor, indicates that larger fish have lower body conditions despite feeding more, and thus support lower levels of endoparasitic infection (Oliveira *et al*., 2016). However, a high abundance of parasites can compromise the body conditions of natural populations (Lizama *et al*., 2007; Morozińska-Gogol, 2015).

The digenean *C. marginatum,* a parasite with low parasitic specificity (Gonçalves *et al*., 2016) which occurred at similar levels of infection in *H. malabaricus* and *H. unitaeniatus* in the present study, was the dominant helminth in the community. The transmission of digenean species is directly related to the food habits of the host, since these endoparasites need more than one host to complete their biological cycle (Pinto *et al*., 2015; Oliveira *et al*., 2016, 2017). In Brazil, in general, metacercaria of *Clinostomum* spp. use *Biomphalaria* spp. mollusks as primary intermediate hosts (Dias *et al*., 2003; Pinto *et al*., 2015), and the *H. malabaricus* and *H. unitaeniatus* of the present study are the secondary intermediate hosts of this endoparasite, with piscivorous birds the definitive hosts (Dias *et al*., 2003; Pinto *et al*., 2015).

The acanthocephalan *G. spectabilis* was found in the intestine, liver, pyloric cecum and mesentery of *H. unitaeniatus*, as well as in the mesentery of *H. malabaricus*, with varying rates of prevalence. However, its greatest abundance occurred in *H. unitaeniatus*, which showed levels of infection similar to those described for this same host from another basin of the Amazon River system (Alcântara & Tavares-Dias, 2015; Gonçalves *et al*., 2016). The life cycle of acanthocephalans involves vertebrate species as definitive hosts and microcrustaceans (amphipods, copepods, isopods and ostracods) as intermediate hosts (Huys & Bodin, 1997). Fish become infected when they prey on microcrustaceans containing acanthella, which can reach the cystacanth and adult stages in *H. malabaricus* and *H. unitaeniatus* in the environment of this study, corroborating the results of Alcântara & Tavares-Dias (2015), for these same host species.

Low levels of infection by *G. s. seriei* were found in *H. unitaeniatus*, indicating that this fish acts as definitive host for this nematode. This species of endoparasite was originally described from *H. unitaeniatus* from French Guiana (Petter, 1975), indicating that these nematodes have a restricted relationship with *H. unitaeniatus,* while *H. malabaricus* is parasitized by *Guyanema baudi* (Weiblen & Brandão, 1992). However, *G. s. seriei* and *G. baudi* use different species of fish as primary and secondary intermediate hosts. This study extends the distribution of *G. s. seriei* to the basin of the Vila Nova River. A high prevalence but low abundance of larvae of *Contracaecum* sp. was found in *H. unitaeniatus* and *H. malabaricus*, although the latter host was less parasitized. However, there was a higher level of *Contracaecum* sp. infection of *H. malabaricus* than *H. unitaeniatus* in another basin in the Amazon River system, due to a larger range of items present in the diet of *H. malabaricus* in the studied environment (Alcântara & Tavares-Dias, 2015; Gonçalves *et al*., 2016). In general, nematodes use microcrustacean species as primary intermediate hosts, while fish may be paratenic, secondary or definitive intermediate hosts (Moravec, 2009; Moreira *et al*., 2009). *Contracaecum* species use piscivorous birds as definitive hosts (Moravec, 2009; Tavares-Dias *et al*., 2014a).

Procamallanus (*S.*) *inopinatus*, a nematode with no parasitic specificity and with wide distribution in Brazil, uses fish species as definitive hosts and species of chironomids as intermediate hosts (Moravec, 1998; Moreira *et al*., 2009; Tavares-Dias *et al*., 2014b; Oliveira *et al*., 2015; Oliveira *et al*., 2016). This nematode was found only in *H. unitaeniatus* and with lower infection levels than those reported for this same host from the Igarapé Fortaleza basin, a tributary of the Amazon River (Alcântara & Tavares-Dias, 2015; Gonçalves *et al*., 2016), a finding probably influenced by the lower availability of intermediate hosts in the environment. However, *H. unitaeniatus* and *H. malabaricus* are the definitive hosts for this endoparasite (Alcântara & Tavares-Dias, 2015). This study extends the distribution of *P.* (*S.*) *inopinatus* to the basin studied.

A high prevalence of *Pseudoproleptus* sp. occurred in *H. unitaeniatus* and *H. malabaricus*, but the highest levels of infection were found in *H. malabaricus*. In the Eastern Amazon region, the larvae of *Pseudoproleptus* sp. were also reported in *Satanoperca jurupari* (Melo *et al*., 2011) and *Aequidens tetramerus* (Tavares-Dias *et al*., 2014a), as cichlid species are possibly part of the diet of *H. malabaricus* and *H. unitaeniatus*, which makes the transmission and development of this nematode even more efficient. *Pseudoproleptus* sp. uses larvae of ephemeral insects and crustaceans as the first intermediate hosts (Moravec, 2007; Moravec & Santos, 2009) while some species of fish act as second intermediate hosts (Moravec and Santos, 2009, Melo *et al*., 2011, Tavares-Dias *et al*., 2014a) and even as a definitive host, such as *H. malabaricus* (Melo *et al*., 2011). This is the first record of *Pseudoproleptus* sp. for *H. unitaeniatus* and extends its geographic distribution to the basin of the Vila Nova River.

In summary, the endoparasites community of *H. malabaricus* and *H. unitaeniatus* was characterized by the predominance of larvae, indicating that these fish are intermediate hosts for most of the parasite species found here. Therefore, these two hosts occupy a central position in the food chain. Finally, the high similarity between the community of endoparasites of *H. malabaricus* and *H. unitaeniatus* indicate a high overlap in environment. There also does not appear to be interspecific competition between the parasites, as they occupy several sites in the host.

Conflict of Interest

Authors state no conflict of interest.

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Case Report

Concurrent infection of a young tourist by hookworm and *Strongyloides stercoralis* during low budget travel in Southeast Asia

M. WESOŁOWSKA[1*], W. RYMER[2], M. KICIA[1], M. POPIOŁEK[3]

[1]Department of Biology and Medical Parasitology, Wroclaw Medical University, J. Mikulicza-Radeckiego 9, 50-367 Wrocław, Poland, E-mail: **maria.wesolowska@umed.wroc.pl*, *marta.kicia@umed.wroc.pl*; [2]Department of Infectious Diseases, Hepatology and Acquired Immune Deficiencies, Wroclaw Medical University, Koszarowa 5, 51-149 Wrocław, Poland, E-mail: *weronika.rymer@gmail.com*; [3]Department of Parasitology, Institute of Genetics and Microbiology, Wroclaw University, Przybyszewskiego 63, 51-148 Wrocław, Poland, E-mail: *marcin.popiolek@uwr.edu.pl*

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Summary

Strongyloidiasis and hookworm infections are neglected helminth diseases widespread in tropical and subtropical areas. In humans, particularly in immunocompromised patients infections potentially may lead to the life-threatening clinical conditions involving the respiratory as well as gastrointestinal systems. The increased number of tourists travelling to tropical regions is associated with more frequent infection with parasites such as *Strongyloides* and hookworm. The infection takes place when filariform larvae penetrate the skin exposed to soil, than migrate through the lungs and finally reach the intestine. Travelers are often not aware of how they could get infected. Physicians may suspect strongyloidiasis and hookworm infections in tourists with diarrhea returning from endemic areas, especially when an elevated eosinophilia is observed. In the literature there are many reports about the presence of parasites in indigenous communities, but very few are available regarding travelers. This paper describes a dual infection with hookworm and *Strongyloides stercoralis* in a young female tourist returning from Southeast Asia. To our knowledge, this is the first report of hookworm and *Strongyloides stercoralis* infection in a tourist from Europe, acquired in an endemic area.
Keywords: *Strongyloides*; hookworms; soil-transmitted helminths; tourists; diarrhea

Introduction

Due to expansions in transport and tourist facilities, an increase in tourist travel has been observed since mid of the 20th century (Gyr, 2010). The number of international tourists increased from 25 million in 1950 to 1186 million in 2015 (UNTWO 2016). International travel can cause risks to the health, and various infections are one of the most common (WHO 2012). Up to 40 % of European tourists have been affected with traveler's diarrhea (TD) (Pitzurra *et al*., 2010) where bacterial and protozoan infections are the predominant causes of TD, however, occasionally helminths are also the causative agent (McGregor AC & Wright 2015).

Hookworms (*Ancylostoma* and *Necator*) and *Strongyloides stercoralis* are endemic intestinal parasites to which travelers may be exposed in countries with warm and humid climates (Tylor *et al.* 2014) and are transmitted through contaminated soil (Ojha *et al*., 2014). Together with *Ascaris* and whipworm they represent a group of parasites named soil-transmitted helminths (STH). Due to the similar way of infection (Hotez *et al*., 2004; Baker *et al*., 2011) hookworms and *Strongyloides* are often spread together. The transmission of the parasite takes place either through direct penetration of the skin by filariform larvae or via the fecal-oral route. (Hotez *et al*., 2004; Taylor *et al*. 2014) *Strongyloides* and hookworms have a very complex life cycle which consists of infective larvae migration through the lungs and finally reach the intestine (Hotez *et al*., 2004; Kassalik & Mönkemüller, 2011). Both, the *Strongyloides* and hookworm adult females produce eggs in the human small intestine. The hookworm eggs deposited by the

* – corresponding author

female are excreted in the feces and mature to its infective stage in the soil, while *Strongyloides* eggs rapidly hatch within the intestinal lumen and release rhabditiform larvae (Ojha *et al.*, 2014; Puthiyakunnon *et al.*, 2014). Most of the *Strongyloides* larvae are discharged by feces, but some of the them transform within the gut into infective filariform larvae (iLF3). After that the larvae can penetrate the end part of the bowel (endoautoinfection) or perianal skin (exoautoinfection) and restart the entire cycle in the human body (Kassalik & Mönkemüller, 2011; Taylor *et al.*, 2014).

Both strongyloidiasis and hookworm infection can present a wide differences in the clinical picture. Starting from abdominal cramping, nausea, and severe diarrhea up to respiratory symptoms and skin reactions (larva currens) (Taylor *et al*. 2014; Forrer *et al*., 2017). High levels of eosinophils are reported for both infections and anemia can also be observed (Buonfrate *et al.*, 2013; Loukas *et al.*, 2016).

During clinical course of infection with *S. stercoralis* many forms of the disease can be observed. It can presented as acute and chronic infection or autoinfection. However, some cases are asymptomatic (Foreman *et al.*, 2006; Olsen *et al.*, 2009). The most severe symptoms are observed in immunocompromised patients caused by malnutrition, HIV infection, glucocorticosteroid therapy, recipients of kidney allograft, as well as the other immunosuppressive factors, where the immune system is no longer able to keep the auto-infective larval cycle under control (Siddiqui & Berk, 2001; Buonfrate *et al.*, 2013; Puthiyakunnon *et al.*, 2014). As a consequence it may cause a severe outcome of strongyloidiasis leading to the life-threatening hyperinfection and dissemination of infection in organs usually not infected during the parasite's normal life cycle (Puthiyakunnon *et al.*, 2014; Tylor *et al.*, 2014). In a such cases, symptoms depend on the organs involved (e.g., liver, heart, lungs, urinary tract, central nervous system). *Strongyloides* filariform larvae also can be responsible for intestinal translocation of bacteria, and subsequent bacteremia, which in immunocompromised patients can be fatal in a few days or weeks (Buonfrate *et al.*, 2013, 2016; Puthiyakunnon *et al.*, 2014).

The decision about the treatment regimen depends on type of infection detected. Albendazole can be used for treatment of hookworm and *Strongyloides* infection where two different therapeutic schemes are utilized. In hookworm the albendazole dose is 400 mg daily for 3 consecutive days; and in strongyloidiasis it is 400 mg twice daily for 7 consecutive days. Regarding the strongyloidiasis, the albendazole is a less effective drug when compared with ivermectin (Tylor *et al.*, 2014; Gilbert *et al.*, 2015). Some authors report of albendazole treatment failure in patient with strongyloidiasis (Boulware *et al.*, 2007; Taylor *et al.*, 2014).

Strongyloides and hookworm infestations are rare in moderate climate zones and primarily occur in tropical and subtropical areas. Particularly those with poor sanitary conditions, where feces are used as a fertilizer or persons who defecate outdoor near fields or gardens (Schär *et al.*, 2014). Hookworms are prevalent in tropical and subtropical climates especially in Africa, Latin America, and Asia. (Ojha *et al.*, 2014). It is estimated that about 740 million people worldwide could become infected by hookworms (Ipankaew *et al.*, 2014). Global prevalence of *Strongyloides* remains unknown, but the World Gastroenterology Organization (WGO) reports that these infections are endemic in the tropics and subtropics. Particularly in Southeast Asia, Latin America, and Sub-Saharan Africa and up to 370 million people are infected worldwide (Tylor *et al*. 2014; Buonfrate *et al*. 2015; WGO, 2017). There are many reports about the presence of parasites in indigenous communities, but very few concerning travelers (Angheben *et al.*, 2011; Ramírez-Olivencia *et al.*, 2014).

Here we report an unusual case of dual infection caused by *Ancylostoma* and *S. stercoralis* in a young tourist and discuss the treatment problems and importance of the medical examination of tourists returning from the endemic areas.

Abbreviations

WBC – white blood cells, Hb – hemoglobin, PLT – platelets, CRP – C-reactive protein, IgE – total immunoglobulin E level

Case presentation

A 23-year-old Polish (Caucasian) woman was admitted to the hospital with diarrhea and abdominal pain. Symptoms started four weeks after her five months trip to the Southeast Asia (Thailand, Myanmar, Laos, and Cambodia. It was a "low budget" trip which involved living in low cost accommodation. She also worked as a waitress and walked barefoot at a beach bar for a few weeks in Cambodia. Apart from diarrhea (five watery bowel movements per day), abdominal pain and mild cough occurred for a few days during hospitalization, and no other clinical symptoms were observed. There were no changes in clinical examination such as lung X-ray examination or abdominal ultrasound. Apart from a high level of eosinophil granulocytes (23.6×10^3 cells/μL, 73.9 % of WBC [norm: 50 – 500 cells/μL, 2 – 5 %]), no other evidence was observed in the laboratory tests (Hb: 13.3 g/dL [norm: 11.5 to 15.5 g/dL] , PLT: 158×10^3 cells/μL [norm: 140 to 440 $\times10^3$ cells/μL], CRP: 1.6 mg/L [norm: <6 ml/L]). Anemia was also not observed.

Fecal specimens were examined for the presence of intestinal parasites at the Department of Biology and Medical Parasitology, Wroclaw Medical University, Poland. Three stool specimens passed at intervals of two days were collected. The samples were examined for parasites by light microscopy and wet smears after formyl-ethyl acetate concentration were performed. Hookworm eggs were found in two fecal samples and eggs measuring 56 – 60 μm in length by 36 – 39 μm in width were discovered (Fig. 1A). Stool examination by Harada-Mori culture (Garcia, 2001) revealed *Strongyloides* rhabditiform larvae. The identification of larvae was based on their biometrics and the morphology. For the most part by the presence of a bulbed esophagus which is shorter than in hookworm rhabditiform larvae, especially the buccal cavity (Fig. 1B).

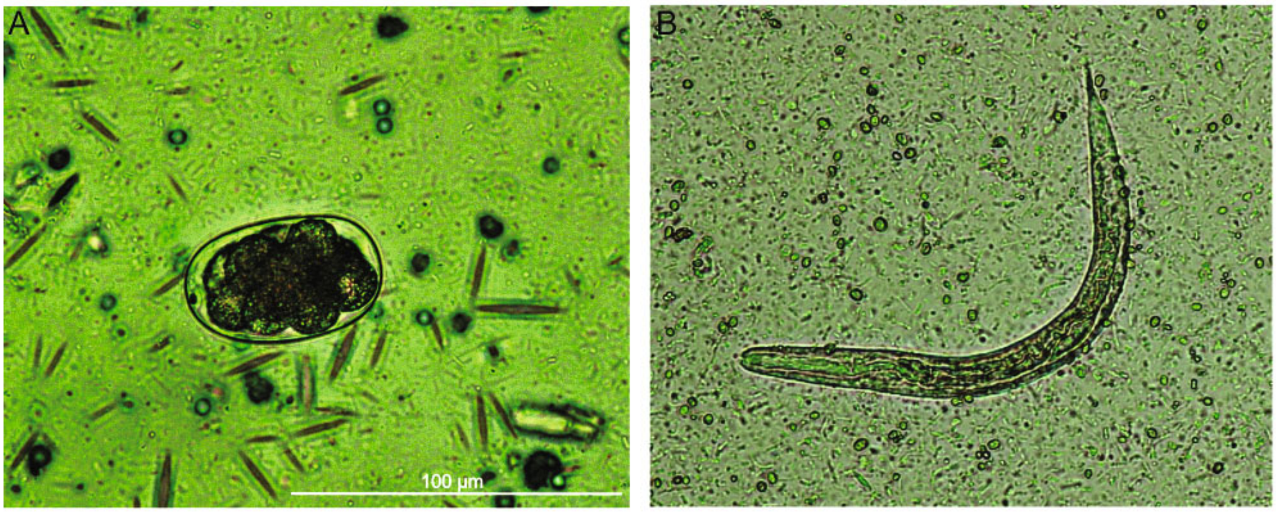

Fig. 1. Wet mounts of feces: panel A depicts hookworm egg measuring 59 µm by 37 µm (×400); panel B shows living rhabditiform larva of *S. stercoralis* (×200). Charcot–Leyden crystals formed from the breakdown of eosinophils are visible in the background. The presence of these crystals in the fecal smear indicates an immune response.

No other parasites were found. Direct fecal smear showed the presence of numerous Charcot-Leyden crystals, which may indicate on an immune response during parasitic infection (Fig. 1 A and B) (Pantanowitz and Balogh, 2004). As soon as the parasite infection (before hookworm infection confirmation) was suspected the albendazole was used for treatment (400 mg twice a day) for seven consecutive days. In two weeks follow-up examination no parasites in feces were observed, and the eosinophil granulocyte levels decreased to 0.88×10^3 cells/µL (15 %).

One month after completing the treatment the symptoms returned. The patient suffered from abdominal pain and had loose stools (up to three per day and some containing mucus). At that time the laboratory examinations showed a higher level of eosinophils (23.4×10^3 cells/µL, 22.5 %). Once again fecal direct smear revealed motile *S. stercoralis* rhabditiform larvae. The patient was repeatedly treated with albendazole (400 mg BID) which was extended up to three weeks). A laboratory examination of the stool specimen (fecal direct smear, formalin-ethyl acetate sedimentation and Harada-Mori culture) after three-week treatment confirmed the elimination of the parasites. The eosinophil granulocyte level decreased to 0.184×10^3 cells/µL (3.28 %).

Three months later loose stools and increased levels of eosinophils (1.539×10^3 cells/µL, 27.0 %) developed again and transient eye edema was observed. No parasites were found in the stool examination as determined by fecal direct smear, formalin-ethyl acetate sedimentation and Harada-Mori culture. For the reason of suspected strongyloidiasis relapse the patient was treated with ivermectin (9 mg once a day for 2 days, repetition of the regimen three months later). Subsequent laboratory studies performed one month and six months after the treatment showed an eosinophil count below 0.5×10^3 cells/µL. All examination of stool samples confirmed the absence of *Strongyloides* infection. Figure 2 present the time frame for the disease symptoms outcome and treatment.

Ethical Approval and/or Informed Consent

Informed consent has been obtained from the patient.

Discussion

Both *S. stercoralis* and hookworms can cause chronic infections, and if not treated properly it can lead to serious clinical complications. We present unusual dual *S. stercoralis* and hookworm infections of a traveler from Poland – a low-prevalence country. The prepatent period and high-risk factors such as barefoot walking suggest that the disease was acquired while staying in Cambodia. The diarrhea appeared four weeks after returning from Asia, which is in line with the parasite life cycle. The average prepatent period [time from penetration of iLF3 to egg stage (hookworm)/ larval stage (*Strongyloides*) appearing in feces] for both infections is about 4 weeks (Puthiyakunnon *et al*., 2014). Since this parasite in our country occurs sporadically and for many years no cases of infection have been recorded it is unlikely that the patient was infected with *Strongyloides* in Poland. Data on *Strongyloides* occurrence in Poland are few and apart. Several cases were recorded in 1976 among brickworks workers and farmers, and one case in an adult person was confirmed in 2006 (Nowak *et al*., 2007). Cambodia is one of the endemic countries for both parasites. The prevalence of hookworm infection among inhabitants of Cambodia varies from 9.6 % to 63.3 % of the population (Inpankaew *et al*., 2014; Schär *et al*., 2014; Yong *et al*., 2014), and prevalence rate for strongyloidiasis is from 2.6 % to 44.7 % (Khieu *et al*., 2013).

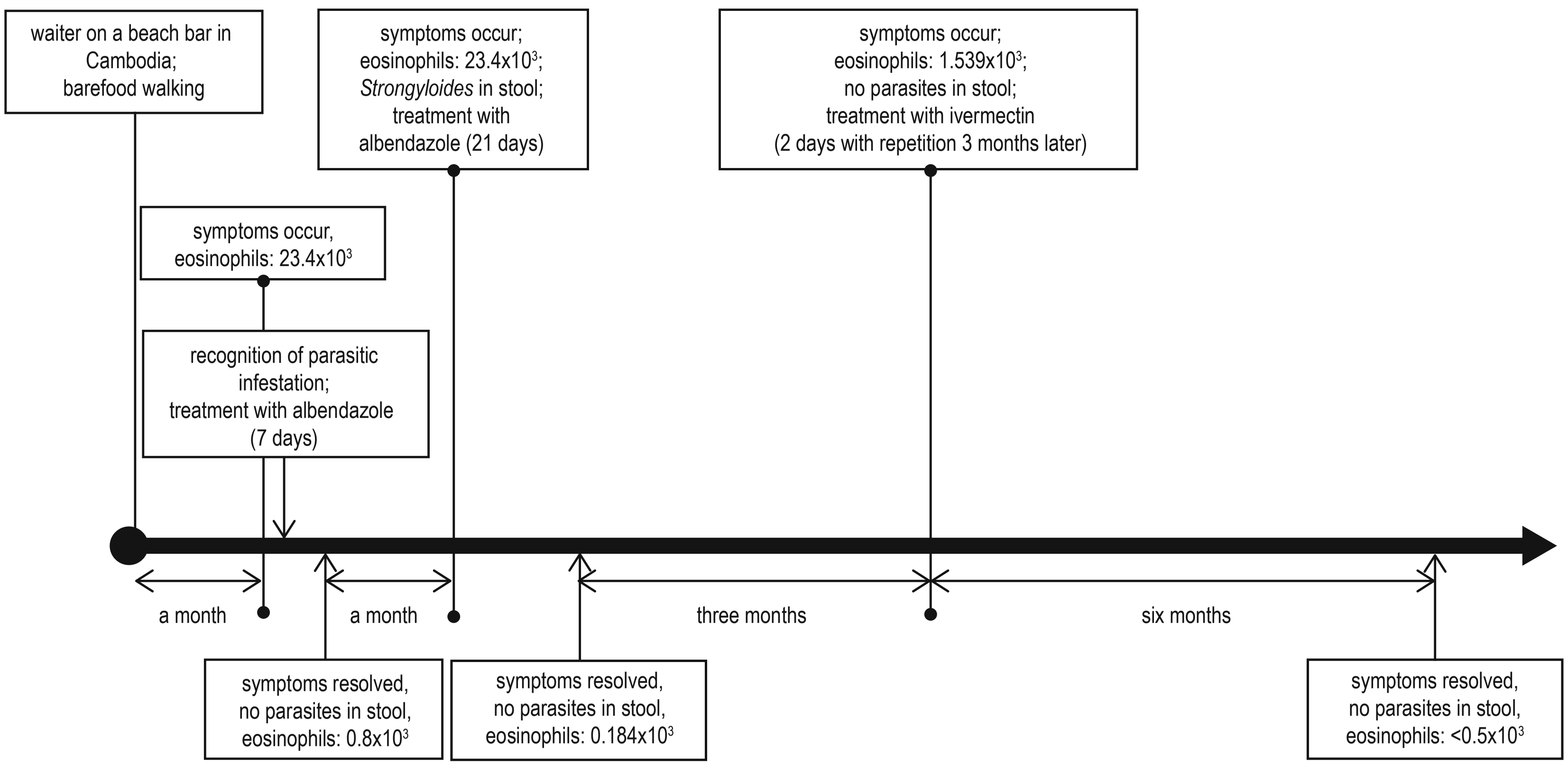

Fig. 2. Dynamics of eosinophils and clinical symptoms prior to and after anthelmintic treatment.

In spite of double infection, oligosymptomatic clinical manifestation and lack of anemia in the described case were observed. We suspect that it was due to a paucity in immunodeficiency factors, the early (invasive) stage of the disease and lack of massive invasion. However, de Silva (2002) in a study on immigrants and refugees with chronic *S. stercoralis* infection in Australia found no correlation between parasite infection and abnormalities in red blood cell morphology or anemia (de Silva *et al.* 2002).

The examination of strongyloidiasis or hookworm infection should be taken into consideration where clinical signs and symptoms, such as diarrhea, eosinophilia or suggestive serologic findings up to at least 1 month after returning from an endemic area occur (Siddiqui & Berk, 2001; Angheben *et al*. 2011; Ramírez-Olivencia *et al*., 2014). The laboratory diagnosis of hookworm and *Strongyloides* is difficult. Negative result does not necessarily imply the absence of infection (Kassalik & Mönkemüller, 2011). Standard diagnostic methods include direct parasite finding as well as culture of stool (e.g. Harada-Mori culture) for the detection of larvae and eggs or serological testing (Siddiqui & Berk, 2001; Ojha *et al*., 2014). Many studies have shown that the analysis of a single stool sample is inadequate and can fail in up to 70 % of cases (Knopp *et al*., 2008; Ojha *et al*., 2014). Repeated stool examinations may increase sensitivity by 50 % and with 3 – 7 stool samples and up to 70 %–100 % (Siddiqui & Berk, 2001; Buonfrate *et al*., 2015) Also, concentration methods such as formalin-ethyl acetate are recommended for the improvement the efficiency of microscopic techniques (Puthiyakunnon *et al*., 2014; Buonfrate *et al*., 2015). A recent study on STH infection diagnosis confirmed high sensitivity of serological methods, which have specificity over 90 % (Siddiqui & Berk, 2001; Buonfrate *et al*., 2013, 2015). Molecular diagnosis is valuable in parasitological investigation, but as yet it is limited by costs and equipment and therefore is not widely available in developing countries (Ojha *et al*., 2014; Buonfrate *et al*., 2015). The detection of concurrent infection seems to be more difficult and may create a bigger problem for diagnostics. In particular when *Strongyloides* rhabditiform larvae occur in feces in small numbers or sporadically (Ojha *et al*., 2014). This suggest that the prevalence rates of dual infection could be underdiagnosed due to insufficient methods as well as small size of the sample (Baker *et al*., 2011; Khieu *et al*., 2014; Forrer *et al*., 2017). It may have effect on false negative results in the diagnostic process (Olsen *et al*., 2009; Puthiyakunnon *et al*., 2014; Taylor *et al*., 2014).

In the present case the treatment of the first infection started as soon as parasite infection was suspected. Because the confirmation of hookworm was later than the initiation of therapy, the patient received empiric albendazole treatment, which was convergent with the alternative regimen for strongyloidiasis (Gilbert *et al*., 2015). However, after the completion of first treatment regimen, the relapse of strongyloidiasis was observed. According to the various studies anthelmintic drugs such as thiabendazole, albendazole and mebendazole, have shown diverse results including treatment failure (Taylor *et al*., 2014). The relapse in this case was probably associated with lower sensitivity of the parasite to albendazole, which has also been noted by the other authors (Boulware *et al*., 2007; Puthiyakunnon *et al*., 2014). The mechanism of albendazole resistance in *Strongyloides* is still not well understood (Taylor *et al*., 2014). Since ivermectin, according to some authors, has shown better elimination rates of about 82 %, this drug is the first choice for therapy of *Strongyloides* infection (Kassalik & Mönkemüller, 2011; Gilbert *et al*., 2015). In Poland there is no registered ivermectin product in a formula for human use. Because obtaining the permission for import from abroad takes a few weeks, it makes our treatment with this drug more problematic. Therefore the patient received prolonged albendazole treatment. We strived to fully eliminate the *Strongyloides* infection, because only the complete eradication of *Strongyloides* allows us to avoid autoinfection and the development of serious illness (Kassalik & Mönkemüller, 2011; Buonfrate *et al*., 2015, 2016). Lawn *et al.* suggest empiric treatment with ivermectin plus albendazole or mebendazole for travelers returning from the tropics, who have present infective diarrhea and eosinophilia without a known cause (Lawn *et al*., 2003). Our case confirms that patient monitoring after the treatment, stool examination and eosinophil count are necessary (three to six months after treatment; due to the risk of treatment failure) to perform. The patient remains under observation, but it seems that the treatment with ivermectin was effective.

Most papers which describe the epidemiology and risk of acquisition of STH infections focus on indigenous people or refugees. The limited studies conducted among tourists returning from endemic areas suggest that the problem of STH infections remain underrated (e.g., German, Belgian Switzerland, Italian and Spanish tourists), (Angheben *et al*., 2011; Ramírez-Olivencia *et al*., 2014; Forrer *et al*., 2017). We suspect it is lower than for indigenous people. However, year by year, the number of international tourists traveling to the STH endemic regions increases. In 2014 Cambodia was visited by more than 3.6 million tourists (Ministry of Tourism of Cambodia, 2016). It may have an influence on the increasing frequency of imported STH infections. As long as the STH infections are not subject to mandatory notification by the sanitary services in the European Union, the real number of infected tourists from low-epidemic countries will remain unknown (Puthiyakunnon *et al*., 2014).

Tourists are often unaware of the risk of STH during their travel and do not know how to keep away from hookworm and *Strongyloides* infections. Barefoot walking is one of the most important risk factors associated with these infection. Coastal regions are among those with the highest rates of transmission. Air-entrained, well-drained sand creates favorable conditions for survival of infective larvae and allows them to take a vertical position for easy attack of the host. The gardens surrounding recreation centers seem to be safe but can also be potentially considered as a risk areas for acquiring strongyloidiasis as well as hookworm infection (Schär *et al*., 2014).

In conclusion, *S. stercoralis* and hookworm infections make important health problems and are major contributors to morbidity and mortality in the developing world (Becker *et al*., 2011). For this reason, tourists should to be informed about the risk of strongyloidiasis and hookworm infections before traveling to the endemic areas. Therefore, physicians should be more aware of the helminth infections presence and focus not only on immigrants from endemic regions, but also on tourists. Failure in treatment of strongyloidiasis, even with the proper therapy should also be taken into account.

Conflict of Interest

Authors state no conflict of interest.

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Research Note

Collyriclosis in Red-backed shrikes *Lanius collurio* from Israel and Czech Republic

V. OSOBA[1]*, J. SEDLÁČEK[2], M. HANZLÍKOVÁ[3], I. LITERÁK[4]

[1]*Faculty of Environmental Science, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Prague 6 – Suchdol, Czech Republic, E-mail: *vlastimil.osoba@seznam.cz*; [2]Institutes of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo náměstí 1, 166 10 Prague 6, Czech Republic, E-mail: *jindrasedl@volny.cz*; [3]Komenského 2, 419 01 Duchcov, Czech Republic, E-mail: *mart.han@seznam.cz*; [4]Department of Biology and Wildlife Diseases, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého 1, 612 42 Brno, Czech Republic, E-mail: *literaki@vfu.cz*

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Summary

One juvenile Red-backed shrike *(Lanius collurio)* with a cutaneous cyst of *Collyriclum faba* under its beak was observed in Israel on 13 October 2016. Another Red-backed shrike (adult female) with multiple cutaneous cysts around the vent was observed in Průhonice, Czech Republic on 19 June 2017. A third Red-backed shrike (adult male) with three cutaneous cysts around the vent was observed in Mariánské Radčice, Czech Republic on 16 July 2017. In the Israeli case, two adult trematodes *C. faba* were found in the cutaneous cyst. In the two Czech cases, *C. faba* was identified indirectly by analysing the cutaneous cyst morphology. *C. faba* had never been recorded previously in Israel.
Keywords: Collyriclosis; Czech Republic; Israel; Red-backed shrike

Introduction

Trematodes *Collyriclum faba* (Bremser in Schmalz, 1831) are parasites of a number of bird species, mainly passerines (Passeriformes) and rarely other birds (e.g. Anseriformes, Galliformes, Apodiformes), in an extraordinarily broad geographical area including some parts of Eurasia and Africa as well as North, Central and South America (Blankespoor *et al.*, 1985; Literák *et al.*, 2003; Literák *et al.*, 2011; Rzad & Busse, 2015; Tahas *et al.*, 2017).

C. faba occurs in pairs within a subcutaneous cyst, the location of which on the host body is the base for distinguishing three ecotypes of *C. faba*: in the femoral or tibial regions (leg ecotype), in the area of the host's vent or in the abdominal area (vent ecotype) or above the coccygeal gland (rump ecotype). Considerably less frequently, the cysts may occur also in the thoracic, sternal and orbital regions and rarely near the eyes and beak (Literák *et al.*, 2003; Literák *et al.*, 2011; Heneberg & Literák, 2013).

Birds are infected in endemic foci, but they often are found outside these foci. Sometimes birds become infected during seasonal migration in different locations while using endemic foci as food sourced (Literak & Sitko, 2006). It is assumed that in Europe the first intermediate host is the small aquatic snail *Bythinella austriaca agg.* (von Frauenfeld, 1857), which occurs focally in the springs of tributaries to the Danube in the Alpine-Carpathian region, and that the second intermediate host is an insect (mayfly) of the family Heptageniidae (Heneberg *et al.*, 2015). The cutaneous cysts are able to develop quickly in 13 – 19 days or less (Literák *et al.*, 2003). In cases of their having smaller numbers of cysts the infected birds may successfully recover, but a larger number of cysts can have fatal consequences for the hosts (Literák *et al.*, 2003; Okulewicz & Sitko, 2012).

In this communication, we describe three new independent cases of collyriclosis in Red-backed shrikes (*Lanius collurio*).

Materials and Methods

One juvenile Red-backed shrike with a subcutaneous cyst at the base of the lower mandible was captured and ringed during

* – corresponding author

the autumn migration at the International Birding and Research Center, Eilat, Israel (29.57N, 34.97E) on 13 October 2016. Its body was thoroughly checked and no other cysts were found. The Red-backed shrike showed no sign of being in poor condition. The shrike was released into the wild after ringing and examination. No additional birds with cutaneous cysts among 163 Red-backed shrikes were observed at that time (from 28 August to 31 October 2017). The netting location in a semi-desert area in a southern part of the Arava Valley is used by migrating birds as a migration stop in the desert.

An adult female Red-backed shrike was captured and ringed in Průhonice, Czech Republic (50.01N, 14.56E) on 19 June 2017. The cysts encircled the vent. Around the close vicinity of the cloaca the cysts were polluted by excrements. Since 2012 there have been mist-netted and ringed at this site 500 – 600 passerines yearly, including seven Red-backed shrikes. No cutaneous lesion had been noted in any previous case.

An adult male Red-backed shrike was captured and ringed at Mariánské Radčice, Czech Republic (50.56N, 13.68E) on 16 July 2017. The bird showed no other pathological changes and was released back into the wild. Since 2011, there have been mist-netted and ringed another 31 Red-backed shrikes at this site, none of which showed any cutaneous lesion.

Ethical Approval and/or Informed Consent

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

In all three cases, cutaneous cysts were identical by shape, colour and size. Also, their structures, including texture of surface and microscopic entry on each cyst, were identical. In the Israeli case, the cyst of size 6 mm was partially necrotic and contained two live oval parasites, the cutaneous trematode *C. faba*. In the second case, the extraordinary cutaneous lesions with rather large numbers of cutaneous cysts were found on this bird (Fig. 1). A total of 16 cysts from 6 to 9 mm in size were localized around the vent and another 6 cysts were around the upper and lower rectrices area. Most of the cysts were with smooth yellow-greyish surface. Each cyst contained a tiny entry hole on its upper central part. In the later case, three cutaneous cysts were found around its vent, each with a tiny visible hole on its upper central part. Sizes of the cysts ranged from 6 to 9 mm.

Discussion

The extent, localization and timing of the cysts' occurrence varied, but they nevertheless were within the characteristic areas known for cutaneous cysts caused by *C. faba* on other bird species, which means typically localized on the vent or rarely near the beak (Literák *et al.*, 2011; Heneberg & Literak, 2013). We concluded that

Fig. 1. Cutaneous cysts of *Collyriclum faba* in *a* Red-backed shrike *(Lanius collurio),* Průhonice, Czech Republic, 19 June 2017. Photographed by Vlastimil Osoba.

the cutaneous cysts observed in the Red-backed shrikes were in all three cases caused by *C. faba*. We report here collyriclosis in Israel for the first time. Formerly, Red-backed shrike was found as a host of *C. faba* only in Tajikistan (Borgarenko, 1984). In that case, 22 Red-backed shrikes were examined and one bird beared one *C. faba* cyst.

Infections with multiple cysts are rare, and they can cause the death of the host (Literák *et al.*, 2003). It has been reported that 86 % of hosts carry only 4 cysts or less (Blankespoor *et al.*, 1985). Only one of the Red-backed shrikes reported here could have suffered from collyriclosis because of the number of cysts found on its body.

In endemic foci of collyriclosis within central Europe, cutaneous cysts of *C. faba* have been found in birds from the end of May to mid-September, with the prevalence peaking in July and August (Literák e*t al.*, 2003). No location, on which Red-backed shrikes with *C. faba* were reported here, is known as an endemic focus of collyriclosis. Moreover, these findings were unique not followed by any similar finding in any of a number of other birds examined at these locations which could indicate a presence of an endemic focus of collyriclosis such as in the Carpathian Mountains (Literák *et al.*, 2003). This situation and the fact, that Red-backed shrike is strictly migrating bird species, are reasons why we suppose that birds in these three cases were infected elsewhere in endemic foci of collyriclosis on their spring or autumn migration routes.

The population of Red-backed shrikes breeding in central Europe including Czech Republic and Slovakia winter in Subsaharan Africa (Cepák *et al*., 2007). Departure of birds from Czech Republic and Slovakia starts in August and their migration route goes southeast through Hungary, Serbia and Greece, then the birds continue to Africa through Egypt. Small portion of birds goes south and it is admitted that they migrate over Apennines as Norwegian birds (Cepák *et al*., 2007). Backwards, they arrive from the south but mostly south-east, using again mainly migration route via the Balkan Peninsula (Tøttrup *et al.*, 2011; Cepák *et al*., 2008; Šťastný *et al.*, 2011).

It seems that the shrikes during migration make frequent but short stopovers, a strategy that requires favourable feeding conditions along the entire migratory route. There are several countries with endemic foci of *C. faba* occurrence that could be used as resting and food source areas for Red-backed shrikes on their migration. The time period for cysts development is still rather unclear, but, assuming it to last several weeks from the beginning of infection, we hypothesize that Red-backed shrikes on their spring migration could be infected in Italy, Slovenia and Austria. These countries have endemic foci of collyriclosis (Brglez, 1977; Govoni *et al.*, 1987; Prosl & Loupal, 1985). The case of collyriclosis in Red-backed shrike from Israel was in a bird that could have been infected during autumn migration in the central European Carpathian foci (Literák & Sitko, 1997; Literák *et al.*, 2003; Literák & Sitko, 2006).

Conflict of interest

Authors state no conflict of interest.

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