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The influence of chestnut wood and flubendazole on morphology of small intestine and lymphocytes of peripheral blood, spleen and jejunum in broiler chickens

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Summary

The study examined subpopulations of lymphocytes in peripheral blood, spleen, and jejunum including morphology of that segment in broiler chicken farm after treatment with flubendazole (Flimabend) and natural extract from chestnut wood (Farmatan). A total of 24 forty-day-old Kalimero-Super Master hybrid chickens were divided into 4 groups (n=6): the Fli group received Flimabend *per os*, 100 mg/g suspension in 1.43 mg of active substance/kg body weight during 7 day of experiment, Far group received Farmatan *per os* at 0.2 % concentration for 6 hours per day during 5 day (experimental days – from 3 to 7); the Far+Fli group received a combination of doses administered in the same way as for the first two groups; and control – C group with no active substance administration. The results demonstrated mild increase of leukocytes, lymphocytes, monocytes, leucocyte common antigen CD45, IgM+ and IgA+ cells in peripheral blood after administration of Flimabend. Similarly, subpopulations of followed lymphocytes (CD3+, CD4+, CD8+, IgM+) were increased in the jejunum after application of that drug. On the other hand, administration of Farmatan revealed opposite effect on determined immunocompetent cells what proves anti-inflammatory effect. Morphology of villi was also negatively influenced by administration of Flimabend. Administration of Farmatan suggests also its preventive administration in chickens. This tanin-containing drug as plant natural product may be used due to its antibacterial activity and as promising alternative to conventional drug with possible antihelminthic effect.

Keywords: Farmatan; Flubendazol; chicken; immunity; intestine

Introduction

Flubendazole is a widely used antihelmintic drug belonging to benzimidazole group. The molecular mechanism of action of flubendazole is based on its specific binding to tubulin. Microtubule-targeted drugs are highly effective for treatment of fungal and parasitic infections (Chatterji *et al*., 2011). They cause disruption of microtubule structure and function, also in the interference with the microtubule-mediated transport of secretory vesicles in absorptive tissues of helminths (Čaňová *et al*., 2017). As the drugs act by disrupting the tubulin-microtubule equilibrium in cells, this resulted to cessation of nutrient transport and eventual cell death. Flubendazole is a potent and efficacious antihelmintic for gastrointestinal nematode infections in poultry and domestic animals (Bradley *et al*., 1983; Tarbiat *et al*., 2016).

Flubendazole is usually administered orally and absorbed through the gastrointestinal tract. The drug is very poorly soluble in aqueous systems, causing its low absorption to the bloodstream and thus very low bioavailability (Michiels *et al*., 1982).

Over the last years, the dietary role of tannins is receiving increas-

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ing interest as they may reduce the number of gastrointestinal parasites in birds (Marzoni *et al*., 2005). Multiple reports suggest the efficacy of tannins or plant extracts in the control of zoonotic bacteria (Tosi *et al*., 2013; Redondo *et al*., 2014) and animal viruses (Lupini *et al*., 2009) in gastrointestinal tract. Tannins can have beneficial effects on the digestion when incorporated into animal diets although their primary mode of action is often not sufficiently known to explain the final in vivo effects (Redondo *et al*., 2014). Schiavone *et al*. (2008) found positive influence on growth performance, especially in young birds at the using up to 0.20 % natural extract of chestnut wood in diet. They did not observe any gross lesions at slaughtering as well as a lack of differences in intestinal length. Those results could indicate that toxicity of chestnut tannin used in their trial was low or absent. On the contrary, severe damage was reported in intestinal wall and other internal organs when doses higher than 30g/kg of tannic acid were administered to chicks (Singleton, 1981).

In the veterinary practice tannins are often use as additives with antihelmintic flubendazole for treatment of birds and to improve animal performance. We suggest that prolong application of flubendazole can be responsible for the modulation of immune response. On the other hand, moderate tannin level could improve health status of poultry.

That's why the goal of the paper is to follow the effect of flubendazole and extract of sweet chestnut (*Castanea sativa* Mill.) on the immunocompetent cells in peripheral blood, spleen and jejunal mucosa. Morphological parameters of intestinal wall evaluated in duodenum and jejunum included height and total area of villi. The potential immunomodulatory and antiparasitical effects are discussed.

Materials and Methods

Chickens and diets

The experiment was conducted in a commercial broiler chicken fattening farm. The broilers were housed in four floor pens identical with the same direction and covered area (0.12 m^2/broiler chicken). Wooden barriers separated the individual groups of chickens from each other. Twenty four chickens 40 days old Kalimero-Super Master hybrid in finisher rearing period were included in the trial. The chickens were weighed, labelled and randomly divided into four groups of 6 chickens each (n=6): C (control), Far (Farmatan®), Fli (Flimabend®) and Far + Fli (Farmatan®+Flimabend®).

The animals had free access to feed and water. The diet corresponded to commercial diet for broiler chickens referred to Feeding Norms for Poultry in Slovakia (code of laws and decrees No. 440/2006). The diet included premix Tekro-finischer, extracted soya starch, wheat, maize, mineral additive. Composition of the diet ($g.kg^{-1}$) was next: Crude protein: 240.84, Crude fat: 52.01, Crude fibre: 35.16; Crude ash: 62.12, Starch: 463.84, Total sugar: 58.13, Reducing sugar: 12.62; Calcium: 8.87; Phosphorus: 9.28; Sodium: 2.58; Methionine: 4.71; Lysine: 13.42; Cystine: 2.90. Chickens were vaccinated against coccidiosis with Livacox Q on 5 day of age.

Administration of preparations and sampling

Extract of sweet chestnut (*Castanea sativa* Mill.) was added into water (Farmatan® liquid, Tanin Sevnica d.d., Slovenia) of Far group chickens in 0.2 % concentration for 6 hours *per day* (8.00 – 14.00) during 5 days (from 3 to 7 experimental day). Chickens of Fli group received individually *per os* antihelminticum flubendazol (Flimabend®, 100 mg/g suspension, KRKA d.d., Slovenia) in 1.43 mg of active substance/kg body weight during 7 days of experiment. Group Far+Fli received Farmatan® liquid and Flimabend® in the similar way and for the same period as groups Fli and Far. Group C served as a negative control and fed with diet without supplementary Farmatan® or Flimabend®.

Two days after the administration of Farmatan® and Flimabend® all chickens were euthanized by intraabdominal injection of xylazine (Rometar 2 %, SPOFA, Czech Republic) and ketamine (Narkamon 5 %, SPOFA, Czech Republic) at doses 0.6 and 0.7 ml/kg body weight, respectively. The samples of peripheral blood were collected before euthanasia by wing vein bleeding from subcutaneous ulnar vein into Heparin (10-20 U/ml PBS, Zentiva, Czech Republic). Remaining samples were collected during the necropsy. Spleen and intestines for flow cytometry and immunohistochemistry were put into phosphate buffered saline (PBS, Sigma, Germany) and for morphometry into 10 % neutral buffered formalin (NBF, Formaldehyd p.a., Centralchem, Slovakia).

White blood cell count

Leukocytes were counted in a haemocytometer using Fried-Lukačová solution (475 µl of solution plus 25 µl of blood). Differential cell counts of 100 cells per slide were done by light microscopy at 1000 magnification using blood smears stained with Hemacolor (Merck, Germany). The total numbers of different subtypes of white blood cells was then calculated: total leukocytes count x proportion of differential cells counted (%) / 100.

Flow cytometry

Peripheral blood lymphocytes were separated by Histopaque gradient sedimentation (1.077 g/mL, Sigma-Aldrich, Germany) according to Boyum (1974). Mouse anti-chicken monoclonal antibodies CD3, CD4, CD8 (T-cells), leucocyte common antigen CD45, IgA and IgM (B-cells) labelled with FITC (SouthernBiotech, USA) were used for immunophenotyping of lymphocytes by direct immunofluorescent method. The control antibody, polyclonal gout-anti mouse FITC-conjugated immunoglobulin $F(ab')_2$ fragment (Dako, Denmark) was used at a working dilution 1:50 with PBS. After separation the lymphocytes were washed twice with PBS. Fifty µL of cellular suspension (1.10^6 lymphocytes in PBS) and 2 µl of specific or control MoAbs were mixed and incubated in dark at 22 °C for 15 min. After being stained, the cells were washed once in 0.5 mL PBS, and resuspended in 0.2 mL of PBS with 0.1 % paraformaldehyde.

Table 1. Absolute count of white blood cells (WBC; $G.L^{-1} = 10^9 . L^{-1}$) in the peripheral blood of chickens (mean ± SD).

WBC	C	Far	Fli	Far+Fli
Leukocytes	13.72 ± 2.71	8.85 ± 2.92[a]	14.58 ± 3.02[b]	14.40 ± 3.46[b]
Lymphocytes	6.68 ± 0.35	5.44 ± 1.57[a]	8.63 ± 1.53[d]	8.37 ± 0.72[d]
Heterophiles	6.23 ± 2.44	2.65 ± 1.41	4.91 ± 2.39	4.63 ± 3.18
Eosinophiles	0.53 ± 0.23	0.47 ± 0.19	0.61 ± 0.34	0.88 ± 0.34
Monocytes	0.43 ± 0.06	0.29 ± 0.07[a]	0.44 ± 0.14	0.46 ± 0.12[b]

Specific superscripts in row indicate significant differences – [ab]P<0.05; [ad]P<0.001

Measurement and analysis of stained cells was performed on FACS system (Becton Dickinson, Germany) provided with a 15 mV argon ion laser. The analysis examined a dot plot of the leukocytes obtained by the forward and side scattering of the physical character of the lymphocyte population. Gates were drawn around lymphocytes based on 90° and forward-angle light scatter. The fluorescence data were collected on at least 10,000 lymphocytes using the Becton Dickinson CellQuest programme. The results are therefore expressed as the relative percentage of the lymphocyte subpopulation which was positive for a specific MoAb. Counting to absolute values was next: absolute count of lymphocytes x relative percentage of subpopulation's lymphocytes/100.

Immunohistochemistry

The jejuna were collected into phosphate-buffered saline (PBS; pH 7.6) and then frozen and cut at –20 °C with Thermo Scientific Cryotome E (Shandon, USA). Frozen 4 µm sections fixed in cold acetone and rinsed in PBS were stained in Shandon Coverplate Technology system (BU Thermo Shandon, Germany). A streptavidin-biotin amplified peroxidase detection system (VECTASTAIN Elite ABC kit, Mouse IgG, PK 6102, Vector Laboratories, USA) was used to detect CD3, CD4, CD8, CD45, IgM and IgA positive lymphocytes. Unlabelled primary mouse anti-chicken monoclonal antibodies (Southern Biotech, USA) for staining CD3 (CD3-UNLB, Clone CT-3, Cat. No. 8200-01), CD4 (CD4-UNLB, Clone CT-4, Cat. No. 8210-01), CD8 (CD8α-UNLB, Clone CT-8, Cat. No. 8220-01),IgA (IgA-UNLB, Clone A-1, Cat. No.8330-01), IgM (IgM-UNLB, Clone M-1, Cat. No. 8300-01) were used in 1:10 dilution with PBS. Mouse IgG1-UNLB antibody (Clone 15H6, Cat. No. 0102-01, Southern Biotech, USA) was used as negative control. All incubations were done at room temperature according to the manual instructions. The sections were rinsed three times with PBS between the two consecutive incubations. The specific colour reaction was developed for 5 min with 3.5 mmol/L 3,3'-diaminobenzidine (DAB, Sigma, Germany), and 30 ppm hydrogen peroxide in200 mmol/L Tris-HCl (pH 7.6). Subsequently the sections were counterstained with haematoxylin and mounted into Pertex (Histolab AB, Swedish). Quantification of labelled lymphocytes was performed under light microscope (NIKON Labophot 2, Germany) at a magnification of × 200 and by using of NIS-Elements version 3.0 software (Laboratory Imaging, Czech Republic). The photos from three jejunal sections at one slide were done and positive staining lymphocytes in 10 randomly chosen areas (a=60 000 μm^2) were counted. Calculation to 1 mm^2 was done as follows: 1 000 000/60 000 x cell numbers.

Histology and morphometry of duodenum and jejunum

Routine histological method with haematoxylin-eosin staining was used. Height and surface area of the villi in duodenal and jejunal samples collected from five chickens were analysed. The histo-

Table 2. Subpopulations of lymphocytes in the peripheral blood (total counts = $G.L^{-1}$) and spleen (relative percentage)(mean ± SD).

Peripheral blood						
Groups	Subpopulations of lymphocytes (mean ± SD)					
	CD3	CD4	CD8	IgM	IgA	CD45
C	2.33 ± 0.79	1.32 ± 0.44	0.80 ± 0.20	0.85 ± 0.24	0.29 ± 0.26	4.32 ± 0.62
Far	2.37 ± 1.08	1.45 ± 0.63	0.72 ± 0.37	0.58 ± 0.23[a]	0.13 ± 0.11[c]	2.89 ± 1.01[a]
Fli	3.12 ± 0.90	1.82 ± 0.71	0.84 ± 0.19	1.20 ± 0.32[c]	0.70 ± 0.39[a]	5.42 ± 1.05[d]
Far+Fli	3.05 ± 0.73	1.99 ± 0.69	0.75 ± 0.18	0.95 ± 0.22	0.26 ± 0.12[c]	5.18 ± 0.84[d]
Spleen						
C	66.14 ± 10.02	16.39 ± 9.27	39.83 ± 7.94	14.88 ± 5.64[a]	25.13 ± 17.25	29.53 ± 9.67
Far	66.29 ± 6.21	24.33 ± 7.24	39.72 ± 10.82	19.91 ± 6.99	16.87 ± 5.95	30.30 ± 5.82
Fli	66.65 ± 5.92	22.59 ± 6.08	39.79 ± 5.62	26.57 ± 7.80[b]	19.97 ± 2.59	32.22 ± 5.90
Far+Fli	63.51 ± 11.10	19.51 ± 8.31	35.17 ± 13.07	18.26 ± 5.67	15.24 ± 3.42	32.76 ± 8.65

Specific superscripts in columns indicate significant differences – [ab]P<0.05; [ac]P<0.01; [ad]P<0.001

Table 3. Subpopulations of lymphocytes in jejunal mucosa (mm^2).

Subpopulations	C	Far	Fli	Far+Fli
CD3	598.90 ± 204.20^{d}	312.00 ± 105.10^{a}	680.10 ± 186.80^{d}	672.30 ± 252.00^{d}
CD4	614.40 ± 187.00^{d}	282.00 ± 94.69^{a}	641.70 ± 185.40^{da}	547.10 ± 151.70^{dc}
CD8	770.10 ± 213.40^{d}	388.60 ± 159.50^{a}	794.20 ± 257.30^{d}	743.20 ± 220.20^{d}
IgM	203.90 ± 122.30^{a}	146.70 ± 91.80^{c}	216.00 ± 88.14^{a}	149.60 ± 73.22^{bc}
IgA	331.10 ± 162.40	250.80 ± 180.40	294.10 ± 200.20	274.60 ± 160.40

Specific superscripts in row indicate significant differences – $^{ab}P<0.05$; $^{ac}P<0.01$; $^{ad}P<0.001$

logical samples were microphotographed (Nikon LABOPHOT 2 with a camera adapter DS Camera Control Unit DS_U2, 4x) and then the NIS-Elements version 3.0 software (Laboratory Imaging, Czech Republic) was used. The heights of the villi were measured from the basal region, which corresponded to the higher section of the crypts, to the apex (μm). Total cutting surface area of separate intestinal segments included length and breadth of villi (μm^2). The data were finally exported to MS Excel and subsequently statistically analysed.

Statistical analysis

Statistical analysis of obtained data was done by one-way analysis of variance (ANOVA) with the *post hoc* Tukey multiple comparison test using GraphPad Software, statistical version 5.0 (USA). The differences between the mean values for the groups of chickens were considered significant when $P < 0.05$. Values were expressed as means ± standard deviation (SD).

Ethical Approval and/or Informed Consent

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

Results

Peripheral white blood cells

Increase in total number of leukocytes (Table 1) was found in Fli and Far+Fli groups when compared with Far group ($P<0.05$). Similarly, the number of lymhocytes was higher in Fli and Far+Fli groups than in Far group ($P<0.001$). Monocytes also demonstrated improved values in Fli and Far+Fli groups in comparison with Far group, but with significance only in Far+Fli group ($P<0.01$).

Phenotyping of lymphocytes in blood and spleen

In the peripheral blood the highest values in all determinated subpopulation of lymphocytes (Table 2) were achieved in Fli group. Its levels outnumbered the counts in C and Far groups with exception to CD4+ cells in combined Far+Fli group. Density of CD3+ and CD4+ (T cells) showed no significant improvement in Fli and Far+-Fli groups. Determination of B cells showed higher values of IgM+ cells in both Fli and Far+Fli groups when compared to Far group, but were significant ($P<0.01$) only for Fli group. IgA+ subpopulation demonstrated highest values ($P<0.01$) not only to Far but also to Far+Fli group. Density of leucocyte common antigen CD45 was increased in Fli and Far+Fli groups when compared with Far group ($P<0.001$).

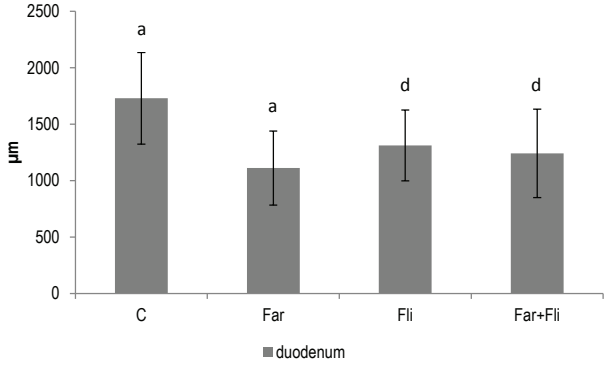

Fig. 1. Height of duodenal villi (mean±SD, $^{ad}P>0.001$).

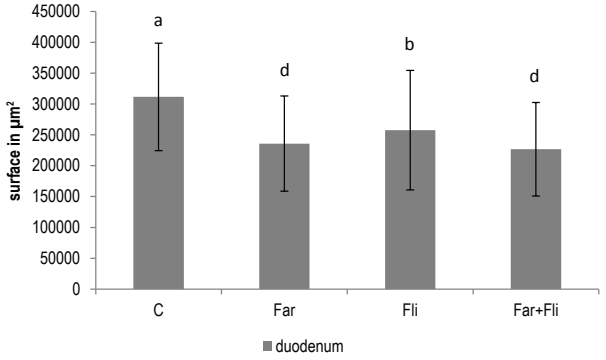

Fig. 2. Cutting surface of duodenal villi (mean±SD, $^{ab}P>0.05$, $^{ad}P>0.001$).

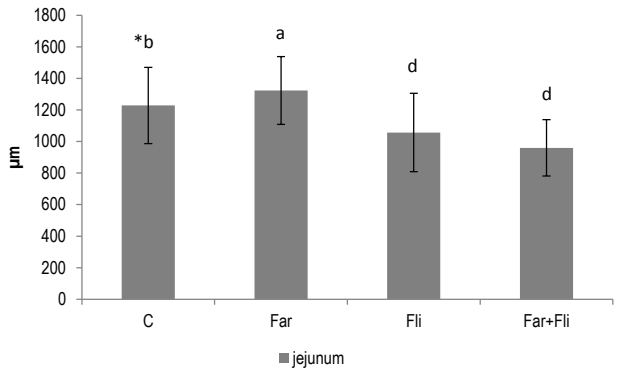

Fig. 3. Height of jejunal villi (mean±SD, $^{ab}P<0.05$,$^{*ad}P<0.001$).

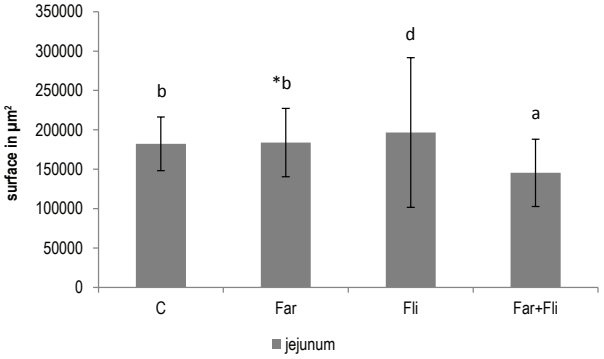

Fig. 4. Cutting surface of jejunal villi (mean±SD, $^{*ab}P<0.05$, $^{ad}P<0.001$).

In spleen (Table 2) the T cell subpopulations (CD3+, CD4+, CD8+) did not changed. Regarding the B cells an increase in IgM+ in Fli group was seen when compared to the control broilers (P<0.05). Density of IgA+ in experimental groups was higher. However, not significant when compared with controls.

Phenotyping of lymphocytes in jejunal mucosa

Jejunal mucosa in Far group (Table 3) showed lower number of CD3+ lymphocytes than seen in Fli, Far+Fli and C broilers (P<0.001). In similar way, the numbers of CD4+ and CD8+ lymphocytes were lower in Far group when compared to Fli, Far+Fli and C groups (P<0.001). Moreover, CD4+ cells were increased in Fli group contrasting to Far+Fli group (P<0.01).

IgM+ cells were found to be lower in Far (P<0.01) and Far+Fli (P<0.05) groups than in C group. In contrast, decrease of IgM+ lymphocytes was found in Far+Fli group in comparison to Fli

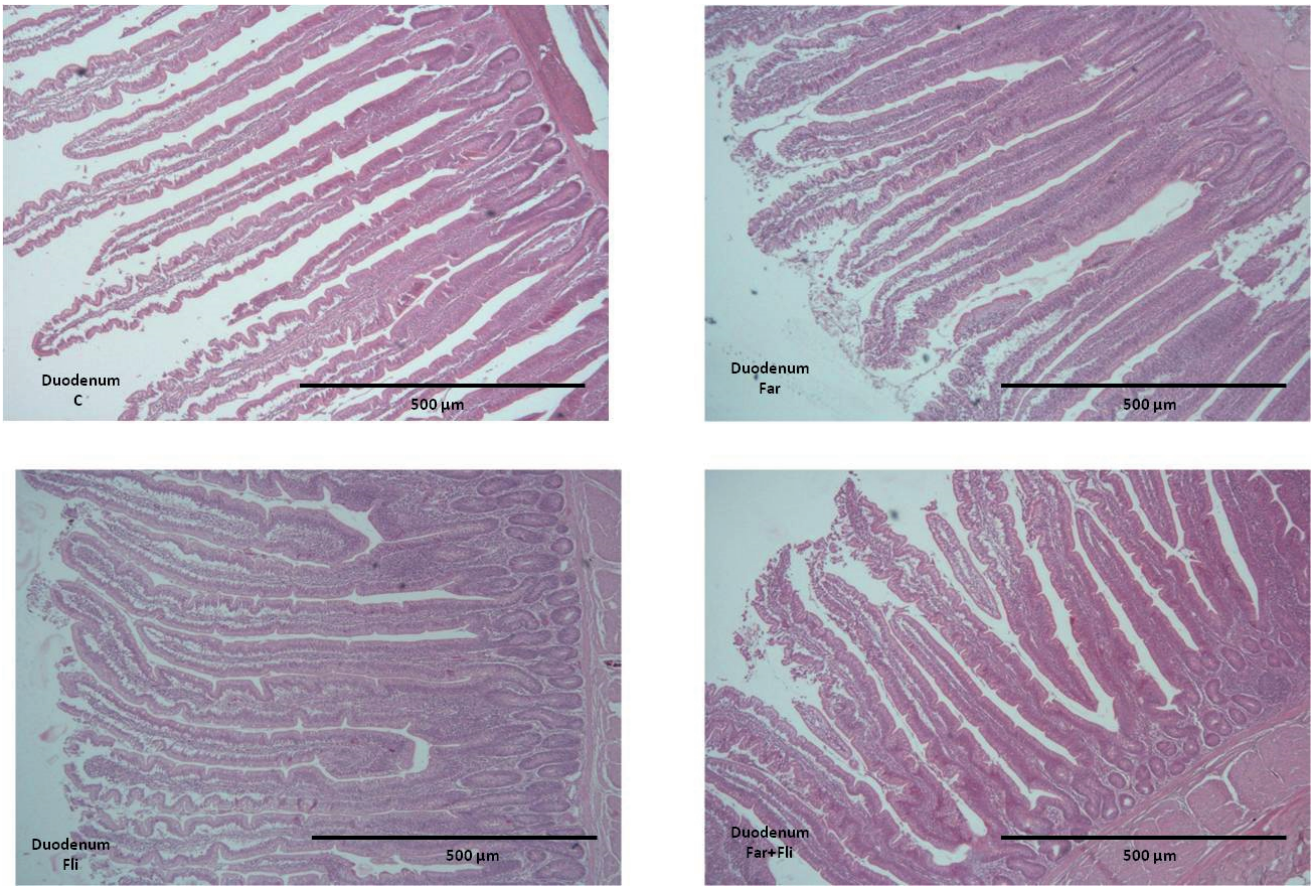

Fig. 5. Histological pictures from duodenum of different chicken' groups (HE stain).

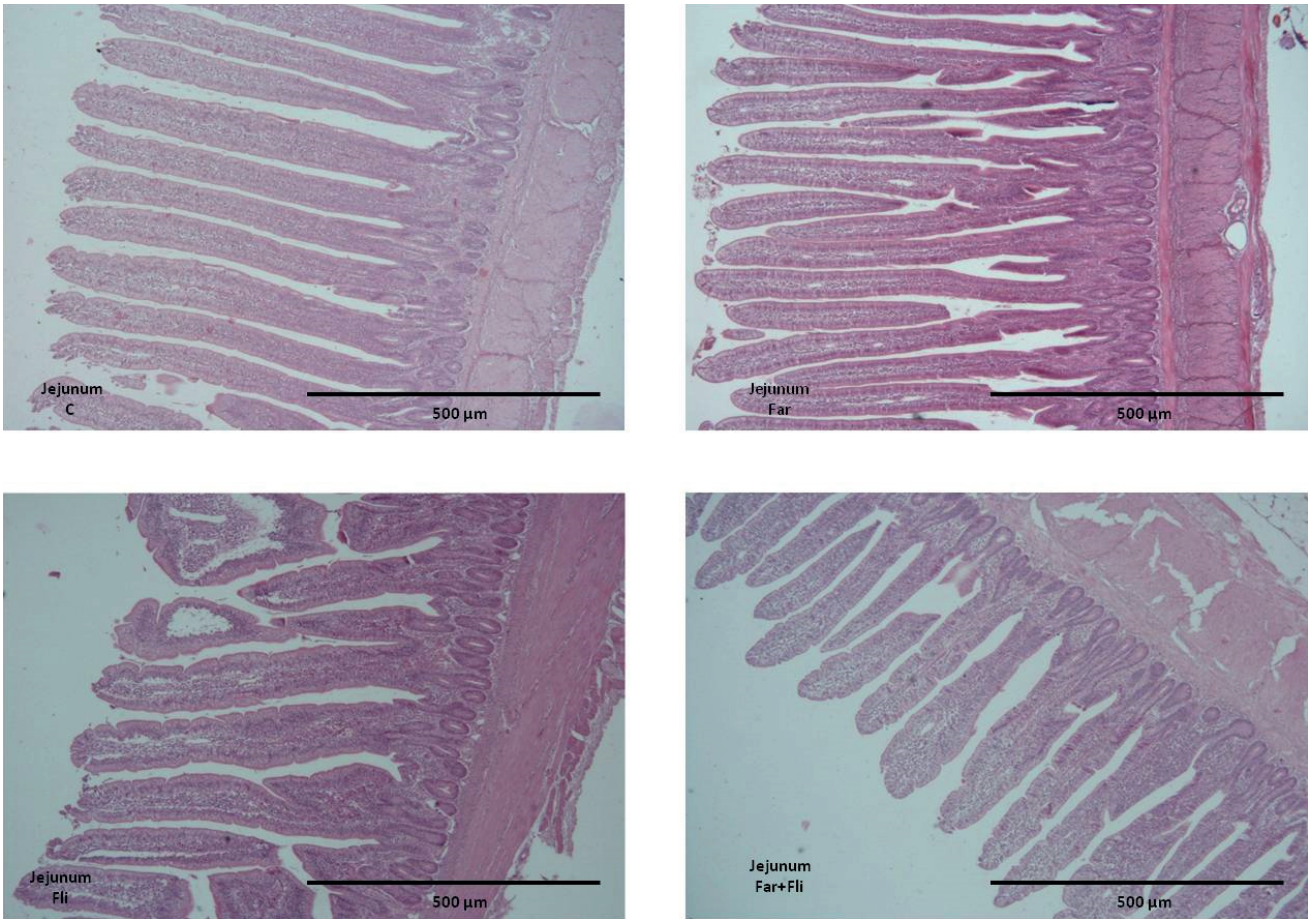

Fig. 6. Histological pictures from jejunum of different chicken' groups (HE stain).

(P<0.01) group. The IgA+ subpopulation was downregulated in experimental group when compared to control broilers.

Morphometry of duodenum and jejunum

In duodenum the Far group showed decrease in height of villi (Fig. 1) as compared with Fli and Far+Fli (P<0.001). Similarly, significant decrease was found between Fli and Far+Fli groups when compared with control group (P<0.001). The highest differences were detected in duodenum (Fig. 5). In comparison to control the cutting surface of duodenal villi (Fig. 2) was lower in Far, Far+ Fli groups (P<0.001) and Fli group (P<0.05).

In jejunum Far and C groups showed increase in height of villi (Fig. 3, Fig. 6) when compared to Fli and Far+Fli groups (P<0.001). However, the Far group outnumbered C group (P<0.05). Cutting surface of jejunal villi (Fig. 4) was lowest in Far+ Fli group in comparison to Fli (P<0.001) and Far groups (P<0.05). Values of Far group were merely higher than values detected in C group (P<0.05).

Discussion

Flubendazole, one of the benzimidazole antihelmintics, is widely used for treatment and prevention of endoparasitic infections in poultry (Baliharová *et al*., 2004). Commercially available flubendazole-based products are used mainly against helminth parasites of chickens as *Ascaridia galli*, *Heterakis gallinarum* and *Capillaria* spp. (Squires *et al*., 2012). On the other hand, tannins included in low concentration of the diet have positive influence on the growth performance (Redondo *et al*., 2014), reduce the spread/occurrence gastrointestinal parasites in birds (Marzoni *et al*., 2005) and was proven to posess antimicrobial activity (Elizondo *et al*., 2010). Beneficial properties of tannins prompted us to test this substance during administration of flubendazole.

Ascaridia galli is a common parasite in small intestine of chickens. After ingestion of the embryonated eggs, these are transported until they reach the duodenum and/or jejunoileum, where they hatch; larvae are released and penetrate the epithelium (Luna-Olivares *et al*., 2015). From these reasons, the evaluation of immunocom-

petent cells and morphological parameters in intestine – jejunum – was selected.

Seven days administration of antihelmintic drug caused mild increase in the number of peripheral blood leukocytes, lymphocytes and monocytes in groups with flubendazole. Similarly, flubendazole modulated the level of IgM+, IgA+ cells and CD45 in peripheral blood, and IgM+ cells in spleen. Observed shift in immunocompetent cells suggests systemic immune response to antihelmintic drug. Our results are in accordance with results done in our laboratory (Karaffová *et al*., 2019) where the upregulation of pro-inflammatory cytokines (IL-1β and IL-18) in Fli group was demonstrated. CD3+, CD4+, CD8+, IgM+ increase in chicken intestine after seven days flubendazole administration support the mild inflammatory role of drug in the chicken *duodenum*. On the other hand, five days administration of farmatan decreased the number of immunocompetent cells in chickens what suggest on tannins anti-inflammatory effect. Recently, many studies demonstrated the anti-inflammatory effect of tannins. Araújo-Neto *et al*. (2010) have shown the anti-inflammatory effect of tannins with *Sideroxylon obtusifolium* on paw oedema induced by carrageenan. Another study exploiting a wound model and skin healing in rats showed its topical anti-inflammatory and healing activity (Leite *et al*., 2015). Finally, anti-inflammatory properties were found in ethyl acetate phase of *Anacardium occidentale* rich in tannins (de Araújo Vilar *et al*., 2016). Recently, Williams *et al.* (2014) provided clear evidence of the direct antihelmintic effects of tannins against *Ascaris*. On the other hand, our working group in another paper (Karaffova *et al*., 2019) demonstrated the upregulation of MUC-2 and IgA gene in the duodenum of chickens after administration of tannin. IgA forms the first line of defence to limit epithelial contact with and penetration by intestinal microbiota and other potentially dangerous antigens (Zhang *et al*., 2015). MUC2 expression increases from anterior to posterior through the gastrointestinal tract (Jiang *et al*., 2013), and protective role of mucins against parasites include the demonstration of trapping of worms in the mucus as well inhibition of parasite motility and feeding capacity (Khan *et al*., 2008). Consistent increase of T cell subpopulations and MUC2 gene upregulation suggest subsequent mucus production in our experiment (Theodoropoulos *et al*. 2001).

Height of villi and cut surface of villi decreased in experimental groups comparing to control. This phenomenon can be explained by lower number of immunocompetent cells in the mucous including villi in Far group and negative effect of benzimidazoles on proliferation of enterocytes. It is known that benzimidazoles can affect also host tubulin (Mackenzie and Geary, 2011) and what can be connected with the decrease the height of villi in groups with benzimidazoles. Increased thickness of depth of crypts in Fli group also suggests mild inflammatory process with increased number of immunocompetent cells.

A normal morphology and intestinal permeability of the small intestine is important to prevent bacteria translocation from the intestinal tract to the body as well as for digestion and absorption of nutrients (Quinteiro-Filho *et al*., 2010; Awad *et al*., 2017). An increase of immunocompetent cells in the peripheral blood and jejunal mucosa in our trial is likely to be indicative of a possible intestinal mucosa barrier dysfunction (Beatty *et al*., 2017) and, consequently bacterial infection. Inflammatory infiltrate is suggests to be contributed to the production of proinflammatory cytokines observed in that experiment (Karaffová *et al*., 2019) and affect on the intestinal epithelium's tight junctions, in turn increasing the mucous permeability to pathogenic bacteria.

In conclusion, the results in our study demonstrated mild inflammatory effect on leukocytes, lymphocytes, monocytes, leucocyte common antigen CD45, IgM+ and IgA+ cells in peripheral blood after administration of Flimabend. Similarly, subpopulations of followed lymphocytes (CD3+, CD4+, CD8+, IgM+) were increased in the intestine after application of that drug. On the other hand, administration of Farmatan revealed the opposite effect on immunocompetent cells what proves to have an anti-inflammatory effect. Morphology of villi and depth of crypts was negatively influenced by administration of Flimabend. Results obtained also suggest the utilisation of Farmatan as preventive – immunomodulatory substance reducing inflammation as well as the adjuvant in treatment with antihelmintics.

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Conflict of Interests

Authors state no conflict of interests.

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Prevalence of soil-transmitted helminths in primary school playgrounds in Edo State, southern Nigeria

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Summary

Schoolchildren in primary schools are mostly at risk of acquiring soil-transmitted helminths (STHs) infections due to their habits (geophagy, onychophagy and playing with barefoot). Profiling soil parasites on school playgrounds is expected to provide an insight to an array of parasites schoolchildren are constantly at risk of acquiring; and this information could guide on intervention programmes. Soil samples from sixteen primary school playgrounds in Edo State (South-South, Nigeria) were collected over a six-month period both in the dry (January, February and March) and wet (May, June and July) seasons in 2018 and early 2019. Samples were processed and analysed following standard parasitological procedures. Of the 576 soil samples collected, 318(55.2 %) were positive with one or more soil parasites. Generally, the predominant parasites recovered from the total number of soil samples collected were: *Ascaris* 127(22 %), *Strongyloides* 111(19.27 %) and hookworm 50(8.68 %). *Ascaris* was most preponderant in the dry season, while *Strongyloides* was the most occurring in the wet season. The mean differences in the parasite load for *Ascaris* and hookworm between dry and wet seasons were not significant; while for *Strongyloides,* it was higher in the wet than dry season. These results could be a consequence of observed poor state of toilet/sanitary facilities as well as the lack or poor state of basic infrastructure like proper drainage and waste disposal systems in the host communities. There is therefore urgent need to interrupt the STHs transmission cycles in the environment and possibly in schoolchildren by instituting sustainable intervention programmes within schools located in STHs endemic regions like southern Nigeria.

Keywords: Schoolchildren; soil-transmitted helminths; seasons, Edo State; southern Nigeria

Introduction

Soil-transmitted helminths (STHs) are a group of intestinal nematode-causing diseases in man. These are roundworm (*Ascaris lumbricoides*), whipworm (*Trichuris trichiura*) and hookworms (*Necator americanus*, *Ancylostoma duodenale* and *A. ceylanicum*). These aforementioned parasites are among the 20 major neglected tropical diseases (NTDs) identified by the World Health Organization (WHO, 2018). The threadworm (*Strongyloides stercoralis*) being an STH, is frequently considered separately because methods used for diagnosis as well as treatment regimen are different (Krolewiecki *et al*., 2013; Puthiyakunnon *et al*., 2014; Buonfrate *et al*., 2015; Forrer *et al*., 2018). STHs are estimated to affect more than 1.5 billion people worldwide (WHO, 2018) with the disability-adjusted life years (DALYs) for *A. lumbricoides* to be between 596, 000-1,290,000, *T. trichiura* (120,000 – 354,000) and hookworms infections (510,000 – 1,340,000) (Global Health Metrics, 2018). The morbidity caused by STHs is commonly asso-

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ciated with the intensity of infection as chronic and repeated infection could lead to malnutrition as well as physical and intellectual growth impairment particularly among school-age children (Bundy, 1995; Hotez *et al*., 2005). Clinical symptoms include: diarrhoea, abdominal pain, nausea, vomiting, cough, fever, dysentery and colitis (Bethony *et al*., 2006).

In addition to the STHs of humans, there are zoonotic STHs (*A. caninum*, *A. braziliense*. *A. suum*, *S. stercoralis*, *T. vulpis*) of domestic animal origin and infection with these parasites could be of public health concern (da Silva *et al*., 2016; Ma *et al*., 2018). For instance, in humans, *A. caninum* can present as eosinophilic enteritis, while *A. braziliense* can cause cutaneous larva migrans without intestinal infection (Prociv and Croese, 1990; Bowman *et al*., 2010). Primary school playgrounds without perimeter fencing in rural communities are likely to be contaminated with zoonotic STHs; and when acquired, these parasites are under-diagnosed due to sundry reasons such as the fact that medical personnel are largely unfamiliar with the identity of such parasites. Although symptoms of zoonotic STHs with human STHs often present very similar clinical pictures, they sometimes require different treatment approaches (Lloyd *et al*., 2014). The incidence of STH infections is dependent on several factors: sanitary, socio-demographic and environmental. The latter comprise the temperature of the soil surface, humidity and precipitation (Weaver *et al*., 2010; Schule *et al*., 2014). In the transmission cycle of STHs, infected humans and animals defecate in the soil, then eggs mature to the infective larvae stage; thereafter, infection could occur on contact with a new host by ingestion or through skin penetration (Schar *et al*., 2013, Hassan *et al*., 2017). In order to become infective, *Ascaris* egg must incubate at 5 to 38°C for 8 to 37 days, *Trichuris* at 5 to 38°C for 20 to 100 days, while for hookworms, it is 2 to 14 days at temperature under 40°C (Brooker *et al*., 2006). Past field studies have shown that soil contaminated with STHs in both rural and urban settings had significant geographic variability (Blaszkowska *et al*., 2011; Nwoke *et al*., 2013; Hassan *et al*., 2017; Oyebamiji *et al*., 2018).

Clearly, STH infections are prevalent in Nigeria and highest burden is seen in the South-West region (Ohiolei *et al*., 2017; Karshima *et al*., 2018). School-age children between ages 5 and14 are reportedly high risk group as a result of their habits (Nwaorgu *et al*., 1998). Many of these primary school children do pick up soil parasites mostly at break/lunch time because during this period, they are engaged in a range of activities that put them at high risk to acquiring infections (Geissler *et al*., 1998). Despite the possible risk of acquiring soil parasitic infections from public primary school playgrounds, there appear to be a dearth of information on the array of possible parasites seen in these areas within school premises in Nigeria. For the first time, we attempt to profile soil parasites in primary schools in Edo State, South-South, Nigeria; and we believe that the availability of these data may raise the level of consciousness/awareness towards understanding the degree to which schoolchildren are exposed and at risk of acquiring STH-infections. This may go a long way to possibly foster interven-

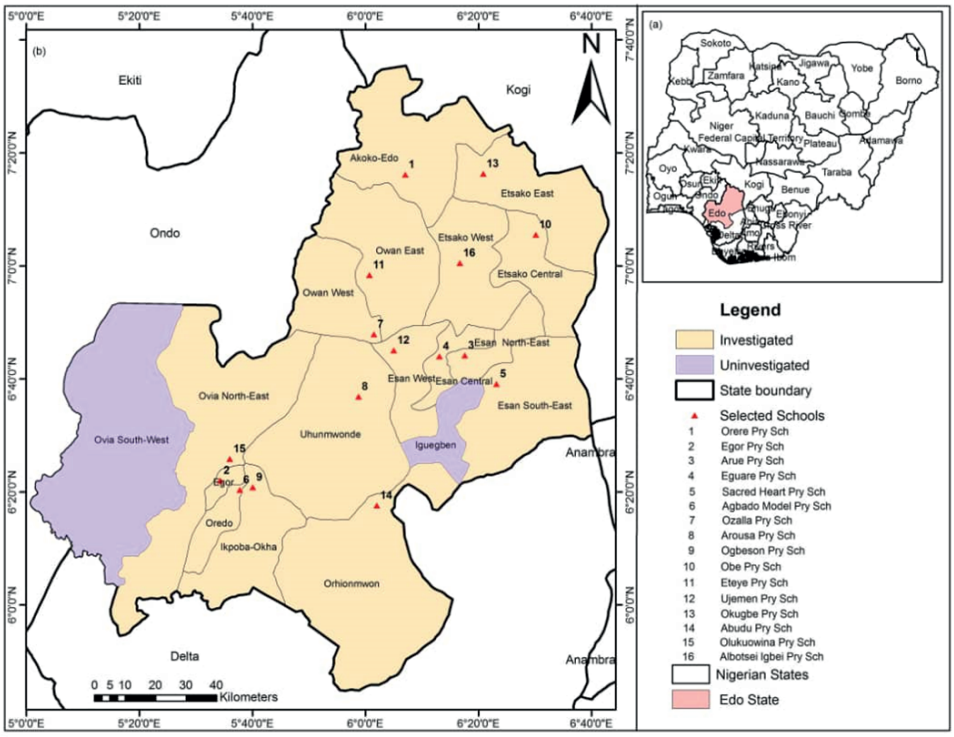

Fig. 1. Map of Edo State indicating study locations.

Table 1. Description of sampled primary schools.

Local Government Area	Primary School	Location	Population size	State of perimeter fencing	Water facility	Toilet facility
Oredo	Agbado pry sch	Urban	1240	Fenced	Borehole	Present
Orhionmwon	Abudu pry sch	Rural	918	Not fenced	Absent	Present
Ovia-North-East	Olukuowina pry sch	Semi-urban	877	Not fenced	Absent	Present
Owan East	Eteye pry sch	Semi-urban	950	Not fenced	Absent	Present
Owan West	Ozalla pry sch	Rural	1294	Not fenced	Absent	Absent
Uhunwonde	Arousa pry sch	Rural	968	Fenced	Present	Present
Etsako West	Albotse Igbei pry sch	Semi-urban	690	Not fenced	Absent	Absent
Ikpobaokha	Ogbeson pry sch	Urban	1099	Fenced	Borehole	Present
Esan North-East	Arue pry sch	Semi-urban	755	Partial fencing	Well	Present
Esan Central	Eguare pry sch	Semi-urban	940	Fenced	Well	Present
Esan South-East	Sacred heart pry sch	Rural	885	Not fenced	Absent	Present
Esan West	Ujemen pry sch	Rural	939	Not fenced	Absent	Present
Etsako Central	Obe pry sch	Semi-urban	978	Fenced	Well	Present
Etsako East	Oghe-Okugbe pry sch	Rural	720	Fenced	Absent	Present
Akoko Edo	Orere pry sch	Urban	1000	Not fenced	Absent	Present
Egor	Egor pry sch	Urban	844	Fenced	Absent	Present

tion programmes. Hence, we collected and analysed soil samples in the months of the year (dry and wet seasons) in which schools were opened for academic activities; and as such, the prevalence and burden of soil parasites in sixteen primary schools were estimated and are thus presented.

Materials and Methods

Study area

Edo State being located in the South-South region of Nigeria comprises 18 local government areas (LGAs) (Fig. 1) with population size of approximately 3,233,366. Its vegetation type is largely rainforest with two seasons: dry (November to March) and wet (April to October). Different communities make up each LGA as each community is likely to have at least one primary school. So, by random selection, one public primary school from each LGA was picked for this study. The settings (urban, semi-urban or rural) where these schools are located, population size, state of water and toilet facilities as well as the state of perimeter fencing around each primary school were noted (Table 1).

Sample collection and processing

In this study, purposive sampling (Jarosz *et al*., 2010) was adopted in selecting areas for soil collection; and as such before any decision on points of soil collection within school premises, we closely inspected these schools at periods when there were breaks from classroom activities which provides for pupils to play around. So, for each school, three locations in which children frequents either to play or carry out other activities were considered for soil collections. Soil samples were collected at three points within each primary school premises twice a month (first and last week of the month) for a six-month period in both dry (January, February and March) and wet (May, June and July) seasons in 2018 and early 2019. We ensured that all-through the period of collection, 100 – 200 g of samples were obtained on same spots within a 5 m radius at 2 – 3 cm depth and preserved at room temperature until needed for parasitological investigation. Soil samples were dried, filtered and weighed to obtain 2 g. Different techniques were used: modified Baermann's (soil samples were suspended in distilled water for 24hrs before examination) and flotation (sucrose solution) methods to obtain eggs and larvae. For the sucrose solution method, faecal samples were first mixed with distilled water, and sieved into tubes to remove large particulates before they were concentrated by centrifugation and decanted. Thereafter, tubes containing the concentrates were refilled with sucrose solution and cover slips placed on the surface of the tubes. Floated eggs/larvae sticks to the surface of the cover slips and these slips are then placed on slides and examined under the microscope (Foreyt, 2001). Using appropriate keys, recovered parasites were identified and also quantified (number of parasites per 2 g of soil) (Horiuchi & Uga, 2013).

Statistical analysis

ANOVA was applied to test differences in monthly variation, while student *t*-test compared variations between seasons for prevalence and parasite density data. Post hoc analysis using Newman-Keuls multiple comparison test was applied to the data on monthly *Strongyloides* density for the dry season. Differences in

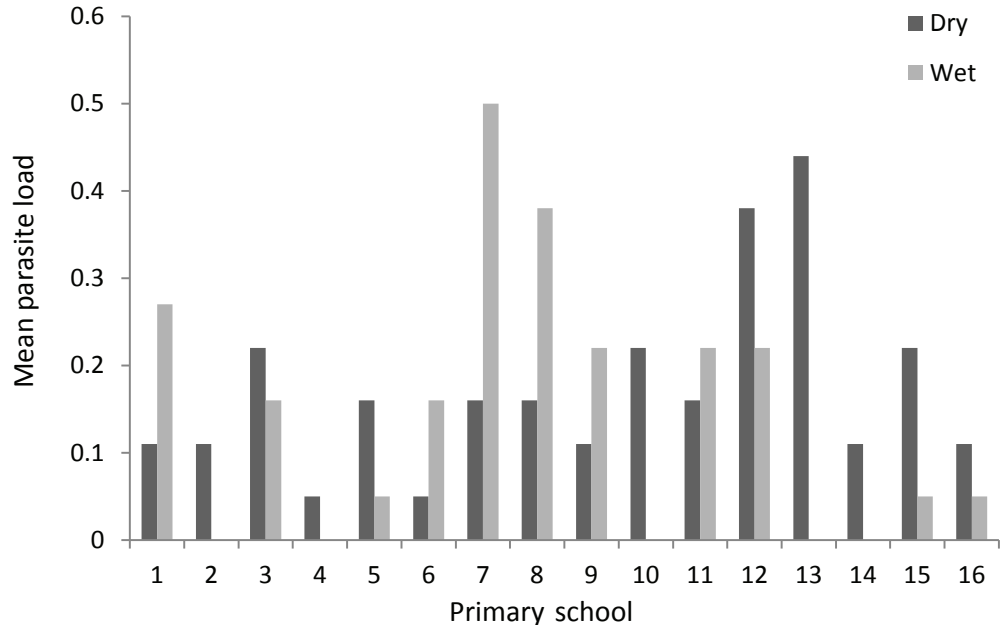

Fig. 2. Seasonal variations with *Ascaris* density.

mean values were significant at $p<0.05$. All analysis was done using GraphPad Prism version 5.01.

Ethics approval and/or Informed Consent

Not applicable.

Results

A total of 576 soil samples were collected in both dry and wet seasons. Four soil parasites were recovered with the following prevalence: *Ascaris* 127(22 %); *Strongyloides* 111(19.27 %); hookworm 50(8.68 %) and *Trichuris* 5(0.86 %). In the dry season, mean values of positive samples was lowest in January (1.87 ± 0.38) and

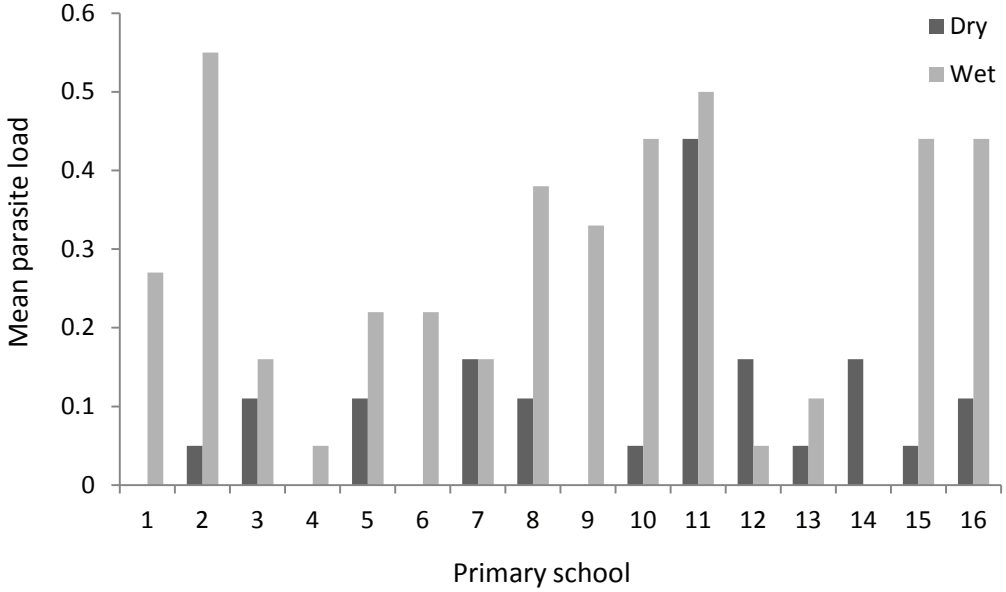

Fig. 3. Seasonal variations with *Strongyloides* density.

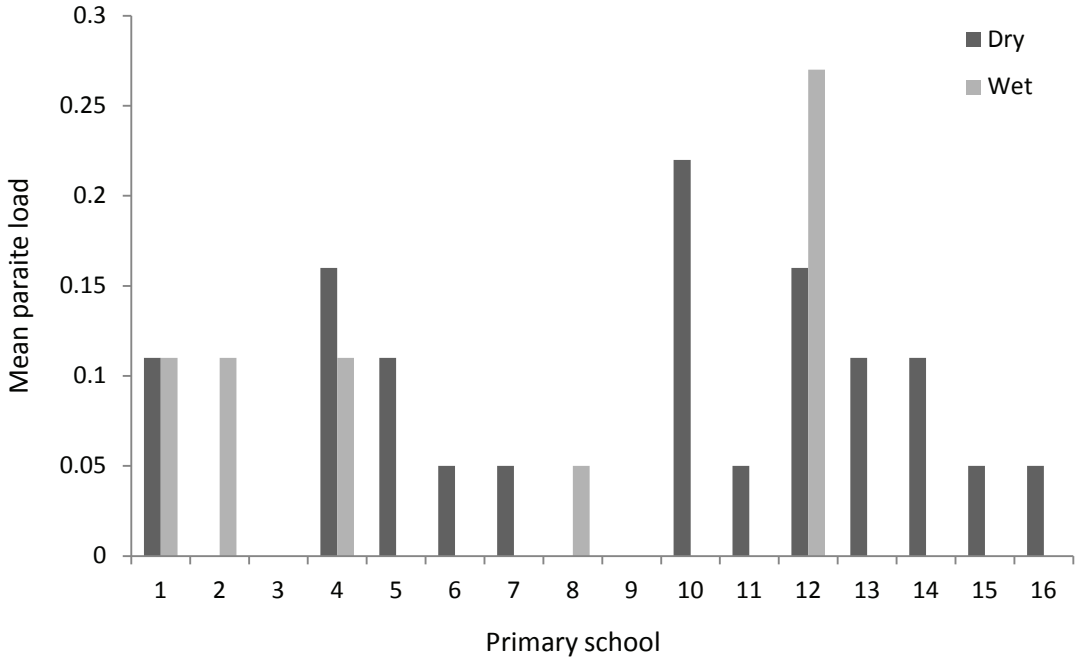

Fig. 4. Seasonal variations with hookworm density.

highest in February (3.75 ± 051) as difference in mean values was significant (F=3.65; p<0.05). In contrast, mean values for the wet season months was not significant (F=0.73; p>0.05). The frequency of positive samples in the dry (2.95 ± 0.3) season was higher than in the wet (3.66 ± 0.29) but mean difference not significant (t=1.61; p>0.05). Of note is the highest number of positive samples recorded in Esan Central and lowest in Owan East. More than 50 % of the total numbers of soil samples were parasite-positive. The least number of positive soil samples were recorded in the month of January, while July samples had the highest number of positives (Table 2).

A total of six parasites were recovered from soil sampled in both seasons, with *Ascaris* being most preponderant in the dry followed by *Strongyloides* and hookworm (Table 3). Meanwhile, in the wet season, *Strongyloides* was the most frequently seen followed by *Ascaris* and hookworm (Table 4). The frequency of occurrence of hookworm in the wet season was higher than in the dry. *Trichuris* parasites were relatively scanty in both seasons.

Nine schools had higher *Ascaris* load in the dry than wet, while seven schools were higher in the wet. Specifically, *Ascaris* density was highest in Etsako Central (0.5 parasite/2 g soil), while in the dry, Etsako West was highest (0.44 parasite/2 g soil) (Fig. 2). The overall mean difference in the *Ascaris* ova density between dry (0.166 ± 0.027/2 g soil) and wet (0.14 ± 0.037/2 g soil) was marginal (95 % CI: -0.078 to 0.11; t=0.36; p>0.05). For *Strongyloides*, twelve of the sites had higher load in the wet than dry season. So far, no positive sample was recorded in one of the primary schools in the wet, while in one study location, the parasite load was same. Only two study locations recorded higher load in the dry than wet (Fig. 3). During the wet season, parasite larvae load was highest in Orhionmwon (0.55 parasite/2 g soil), while in the dry, it was highest in Esan South-East (0.44 parasite/2g soil) (Fig. 2). In the dry season, parasite load was significantly reduced in the month of January; and by post hoc analysis, significant difference was between January and February (q=3.69). The overall mean difference between dry (0.1 ± 0.027/2g soil) and wet (0.26 ± 0.44/2g soil) was significant (95 %CI: -0.27 to -0.059; t=3.19; p<005). For hookworm, of the sixteen (16) sites sampled, only five (5) were positive in the wet season, while twelve (12) sites were positive in the dry. Hookworm ova density in the wet season was highest in Esan West (0.27parasite/2g soil), while in the dry, it was highest in Esan North-East (0.22 parasite/2g soil) (Fig. 4). Meanwhile, overall mean difference between dry (0.076 ± 0.016/2g soil) and wet (0.04 ± 0018/2g soil) was not significant (95 %CI=-0.015 to 0.087; t=1.44; p>005).

Discussion

Most of the efforts by WHO to break the cycles of STH transmission is primarily focused on mass drug administration but has posited that this goal is not achievable without deploying environmental measures to interrupt acquisition of new infections (Anderson *et al*., 2014; Truscott *et al*., 2014). Identification and estimating soil parasites burden could be in part the first step towards achieving this goal as information on the profile of dominant soil parasites in any locality could be useful in a complementary manner in planning an effective and sustainable preventive and control programme. More than half of the samples collected were positive of

Table 2. Frequency of occurrence of parasite-positive soil samples across primary school playground during the dry and wet seasons.

Local Government Area	Primary school	Positive soil sample (dry season)			Total	Positive soil sample (wet season)			Total	Grand total
		January	February	March	n(%)	May	June	July	n(%)	N(%)
Oredo	Agbado pry sch	2	0	6	8(44.44)	6	4	6	16(88.88)	24(66.66)
Orhionmwon	Abudu pry sch	0	6	2	8(44.44)	6	6	2	14(66.66)	22(61.11)
Ovia-North East	Olukuowina pry sch	2	6	0	8(44.44)	2	4	6	12(66.66)	20(55.55)
Owan East	Eteye pry sch	2	4	0	6(33.33)	4	0	2	6(33.33)	12(33.33)
Owan West	Ozalla pry sch	4	6	2	12(66.66)	4	0	6	10(55.55)	22(61.11)
Uhunwonde	Arousa pry sch	0	0	2	2(11.11)	2	6	2	10(55.55)	12(33.33)
Etsako West	Albotse Igbei pry sch	2	4	4	10(55.55)	4	4	4	12(66.66)	22(61.11)
Ikpobaokha	Ogbeson pry sch	0	4	6	10(55.55)	4	2	6	12(66.66)	22(61.11)
Esan North- East	Arue pry sch	2	4	0	6(33.33)	6	2	6	14(77.77)	20(55.55)
Esan Central	Eguare pry sch	4	4	6	14(77.77)	6	4	4	14(77.77)	28(77.77)
Esan South-East	Sacred heart pry sch	2	6	2	10(55.55)	2	4	6	12(66.66)	22(61.11)
Esan West	Ujemen pry sch	4	4	2	10(55.55)	2	4	6	12(66.66)	22(61.11)
Etsako Central	Obe pry sch	4	4	6	14(77.77)	0	4	2	6(33.33)	20(55.55)
Etsako East	Oghe-Okugbe pry sch	0	4	6	10(55.55)	0	0	0	0	0
Akoko Edo	Orere pry sch	2	0	2	4(22.22)	6	6	4	16(88.88)	20(55.55)
Egor	Egor pry sch	0	4	6	10(55.55)	4	2	4	10(55.55)	20(55.55)
Total		30(31.25)	60(62.5)	52(54.16)	142(49.3)	58(60.41)	52(54.16)	66(68.75)	176(61..11)	318(55.2)

[n(%); n= number of positive samples by season; %= percentage of positive samples against total number of collected samples by season]; [N(%); N=total number of positive samples for both wet and dry seasons; %=percentage of positive samples against total number of collected samples in both dry and wet seasons]

Table 3. Prevalence of soil parasites in the dry season.

Local Government Area	Primary school		Positive soil sample			Parasites			
			January	February	March	*Ascaris* (ova) n(%)	*Strongyloides* (larvae)	Hookworm (ova) n(%)	*Trichuris* (ova) n(%)
		1	-	-	Hookworm				
Oredo	Agbado pry sch	2	-	-	*Ascaris*	2(11.11)	-	4(22.22)	-
		3	-	-	Hookworm; *Ascaris*				
		1	-	*Ascaris*	-				
Orhionmwon	Abudu pry sch	2	-	-	*Ascaris*	3(16.66)	3(16.66)	-	-
		3	-	*Strongyloides*	-				
		1	-	*Strongyloides*	-				
Ovia North-East	Oluku Owina pry sch	2	-	*Strongyloides*; *Ascaris*	-	4(22.22)	4(22.22)	-	-
		3	*Ascaris*	*Ascaris*	-				
		1	Hookworm	Hookworm	-				
Owan East	Eteye pry sch	2	-	-	-	2(11.11)	-	4(22.22)	-
		3	-	*Ascaris*; Hookworm	-				
		1	-	*Strongyloides*; Hookworm	-				
Owan West	Ozalla pry sch	2	*Ascaris*	Hookworm	-	5(27.77)	3(16.66)	2(11.11)	-
		3	*Ascaris*	*Ascaris*; *Strongyloides*	*Ascaris*				
		1	-	-	-				
Uhunwonde	Arousa pry sch	2	-	-	-	1(5.55)	-	1(5.55)	-
		3	-	-	*Ascaris*; Hookworm				
		1	-	-	-				
Etsako West	Aibotse Igbei pry sch	2	-	*Ascaris*	*Strongyloides*;Hookworm	4(22.22)	4(22.22)	2(11.11)	-
		3	*Ascaris*	*Ascaris*	*Strongyloides*				
		1	-	*Ascaris*	*Strongyloides*				
Ikpobaokha	Obeson pry sch	2	-	*Ascaris*	*Ascaris*	7(38.88)	3(16.66)	-	-
		3	-	-	*Ascaris*				
		1	-	*Trichuris*	-				
Esan North- East	Arue pry sch	2	-	*Ascaris*	-	4(22.22)	-	-	2(11.11)
		3	*Ascaris*	-	-				

		1	Hookworm	Hookworm	*Strongyloides*				
Esan Central	Eguare pry sch	2	-	Hookworm	*Ascaris*	7(38.88)	3(16.66)	4(22.22)	-
		3	*Ascaris*	-	*Ascaris*				
		1	Hookworm	*Strongyloides*	-				
Esan South-East	Sacred heart pry sch	2	-	*Strongloides*	-	4(22.22)	4(22.22)	2(11.11)	-
		3	-	*Ascaris*	*Ascaris*				
		1	*Ascaris; Strogyloides*	-	-				
Esan West	Ujemen pry sch	2	-	*Strongyloides*	-	4(22.22)	3(16.66)	3(16.66)	-
		3	*Ascaris*	Hookworm	Hookworm; *Ascaris*				
		1	-	Hookworm	*Strongyloides*				
Etsako Central	Obe pry sch	2	*Ascaris*	*Ascaris*	*Ascaris*	10(55.55)	2(11.11)	2(11.11)	-
		3	*Ascaris*	-	*Ascaris*				
		1	-	Hookworm	*Strongyloides*				
Etsako East	Oghe-Okgbe pry sch	2	-	-	*Strongyloides*	2(11.11)	5(27.77)	3(16.66)	-
		3	-	*Ascaris*; Hookworm	*Strongyloides*				
		1	-	-	-				
Akoko-Edo	Orere pry sch	2	Hookworm; *Ascaris*	-	*Strongyloides*	1(5.55)	2(11.11)	1(5.55)	-
		3	-	-	-				
		1	-	*Strongyloides*	*Ascaris*				
Egor	Egor pry sch	2	-	*Strongyloides*	Hookworm	3(16.66)	5(27.77)	2(11.11)	-
		3	-	-	*Ascaris*; *Strongyloides*				
Total N(%)						70(24.3)	41(14.26)	30(1.04)	1(0.34)

[n(%); n=number of samples positive for respective parasite in each primary school; %=percentage of samples positive for respective parasites in each primary school against total number of collected samples in the surveyed primary school); [N(%); N=total number of samples positive for respective parasite across the sixteen primary schools; %= percentage of samples positive for respective parasite across the sixteen primary schools against the total number of collected soil samples in the sixteen primary schools]

Table 4. Prevalence of soil parasites in the wet season.

Local Government Area	Primary school	Positive soil sample				Parasites			
			May	June	July	*Ascaris* (ova) n(%)	*Strongyloides* (larvae)	Hookworm (ova) n(%)	*Trichuris* (ova) n(%)
Oredo	Agbado pry sch	1	*Ascaris*; *Strongyloides*	*Ascaris*	*Strongyloides*	3(16.66)	7(38.88)	6(33.33)	-
		2	Hookorm	*Strongyloides*	Hookworm				
		3	Hookworm	-	*Strongloides*; *Ascaris*				
Orhionmwon	Abudu pry sch	1	*Strongyloides*; Hookworm	*Strongyloides*	-	-	10(55.55)	2(11.11)	-
		2	*Strongyloides*	Hookworm	*Strongyloides*				
		3	*Strongloides*	*Strongyloides*	-				
Ovia North-East	Oluku Owina pry sch	1	-	-	*Strongyloides*; *Ascaris*	5(27.77)	2(11.11)	2(11.11)	-
		2	*Ascaris*	*Ascaris*	-				
		3	-	-	Hookworm				
Owan East	Eteye pry sch	1	-	-	-	-	2(11.11)	1(5.55)	-
		2	Hookworm	-	-				
		3	*Strongyloides*	-	-				
Owan West	Ozalla pry sch	1	*Ascaris*	-	*Strongyloides*	3(16.66)	5(27.77)	-	-
		2	*Strongyloides*	-	*Strongyloides*				
		3	-	-	-				
Uhunwonde	Arousa pry sch	1	-	*Ascaris*	-	4(22.22)	4(22.22)	-	-
		2	-	*Ascaris*	-				
		3	-	*Strongyloides*	*Strongyloides*				
Etsako West	Aibotse Igbei pry sch	1	-	*Strongyloides*	*Ascaris*	5(27.77)	3(16.66)	-	-
		2	*Ascaris*	-	*Ascaris*				
		3	*Ascaris*	*Strongyloides*	-				
Ikpobaokha	Obeson pry sch	1	*Ascaris*	-	*Ascaris*; *Strongyloides*	7(38.88)	4(22.22)	1(5.55)	-
		2	*Ascaris*	*Ascaris*	*Strongyloides*; Hookworm				
		3	-	Ascaris	-				

		1	*Strongyloides*; *Ascaris*	-	*Strongyloides*; *Ascaris*				
Esan North- East	Arue pry sch	2	*Ascaris*	*Trichuris*; *Strongyloides*	*Strongyloides*; *Ascaris*	7(38.88)	6(33.33)	-	1(5.55)
		3	*Ascaris*	-	*Strongyloides*				
		1	*Strongyloides*	*Strongyloides*	-				
Esan Central	Eguare pry sch	2	*Strongyloides*; Hookworm	-	*Strongyloides*	2(11.11)	7(38.88)	2(11.11)	3(16.66)
		3	*Trichuris*; *Ascaris*	*Trichuris*	*Strongyloides*				
		1	-	-	*Strongyloides*				
Esan South-East	Sacred heart pry sch	2	*Ascaris*	*Ascaris*	*Strongyloides*	6(33.33)	4(22.22)	2(11.11)	-
		3	-	*Ascaris*	*Strongyloides*				
		1	-	*Strongyloides*	*Ascaris*; Hookworm				
Esan West	Ujemen pry sch	2	-	-	*Ascaris*	5(27.77)	3(16.66)	4(22.22)	-
		3	Hookorm	Hookworm	*Ascaris*				
		1	-	-	-				
Etsako Central	Obe pry sch	2	-	*Strongyloides*	-	2(11.11)	3(16.66)	-	-
		3	-	*Ascaris*	-				
		1	-	-	-				
Etsako East	Oghe-Okgbe pry sch	2	-	-	-	-	-	-	-
		3	-	-	-				
		1	*Ascaris*	*Ascaris*	-				
Akoko-Edo	Orere pry sch	2	-	*Strongyloides*	*Strongyloides*	6(33.33)	8(44.44)	-	-
		3	*Strongyloides*	*Strongyloides*	*Strongyloides*				
		1	-	-	-				
Egor	Egor pry sch	2	-	-	-	2(11.11)	2(11.11)	-	-
		3	*Ascaris*	-	*Strongyloides*				
			Total N(%)			57(19.79)	70(24.3)	20(6.94)	4(1.38)

[n(%); n=number of samples positive for respective parasite in each primary school; %=percentage of samples positive for respective parasites in each primary school against total number of collected samples in the surveyed primary school); [N(%); N=total number of samples positive for respective parasite across the sixteen primary schools; %= percentage of samples positive for respective parasite across the sixteen primary schools against the total number of collected soil samples in the sixteen primary schools]

one or more parasites as this could be an indication of the level of soil contamination. In January, positive samples were at the lowest but increased in subsequent months. In the state, usually in the month preceding January (December), there are no rains, while in February and March, the state experiences both its first and in some areas, the second rainfall for the year. Meanwhile, by May, the rainy season fully commences and peaks in July. Rainfall comes with high humidity and lower temperatures (23 and 30°C) and these conditions favour the presence and development of soil parasites (Brooker *et al*., 2006), while temperatures from 35°C and above which is often the case in December and January could potentially disintegrate parasites (Rocha *et al*., 2011; Steinbaum *et al*., 2016). Further, in these areas, improper disposal of human and animal faeces is common practice because a majority of these communities where these schools are located lack proper drainage and waste disposal systems. Therefore, when it rains and get flooded, most of these playgrounds could receive faecal-contaminated water from the surrounding environment, partly influencing the rise in the prevalence of STH eggs and larvae on playgrounds (Echazu *et al*., 2015).

The predominant parasites in these areas were *Ascaris*, *Strongyloides* and hookworm, while the occurrence of *Trichuris* was relatively scanty. So we believe that one of the sources of soil contamination with STHs in these playgrounds may have been through open defecation from pupils and inhabitants of respective host communities living close to the school premises. Pit-toilet type was seen in all the schools and shared by pupils. Many pupils have apathy towards the use of a common toilet facility due to it often unhygienic state as they are poorly managed. A report has shown that shared latrines are likely to be dysfunctional, less clean and have flies and faeces littered; and that people who shared latrines were more likely to practice open defecation (Heijnen *et al*., 2015). Another source of contamination could be as a result of lack/partial perimeter fencing, whereby animals could freely move into and out of the school premises and defecate. Coprophagia of human faeces by dogs increases the possibility of transporting STH eggs into the playground as sticky-coated *Ascaris* egg might adhere to the dog's coat for relatively longer period (Nonaka *et al*., 2011; Traub *et al*., 2002). Aside being reservoir hosts, the role of dogs in the transmission cycle of *Ascaris* has been suggested (Shalaby *et al*., 2010). However, it is difficult to differentiate *S. steroralis* larvae, hookworm eggs and larvae as well as *T. vulpis* eggs from the human-infecting STH species deposited possibly by dogs on the playgrounds. Whichever the case, any of these parasites can potentially cause human infection. Meanwhile, a survey of the intestinal parasites of sheep, goat, cattle and dogs across the states possibly indicate high incidence of zoonotic parasites and these could be of public health importance to these children (unpublished data) as reported elsewhere (Shalaby *et al*., 2010; Areekul *et al*., 2010; Steinbaum *et al*., 2019; Pipikova *et al*., 2017). The prevalence and load of *Ascaris* and hookworm between the dry and wet seasons were similar; while for *Strongyloides*, positive samples were higher in the wet than dry. So seasonal variation in parasite's prevalence and burden demonstrates period of higher or lower risk of infections as well as changes that may have occurred to the source(s) of contamination over time (Wong & Bundy, 1990). The optimal conditions for *S. stercoralis* to thrive in an environment include soil temperatures between 20 to 28ºC and high moisture; which is likely the case during the wet seasons in most parts of southern Nigeria as larvae dies rapidly under unfavourable conditions. However, evidence has shown that geographical and climatic conditions are not the primary factors determining the disease presence but rather the level of infrastructural facility, sanitation and socioeconomic status; and as such, strongyloidiasis is recently referred to as: ''a disease of disadvantage and poor sanitation'' (Beknazarova *et al*., 2016). The overall sanitary conditions as well as the state of the infrastructural facilities in most of the schools are poor; but specifically, these primary schools (Abudu and Sacred heart) with relatively high load of *Strongyloides* lacked perimeter fencing.

Detection of STHs in children playground suggests that children exposure to the soil poses substantial health risk. Geophagy is widespread among school children and not limited to toddlers, infants and pregnant women; and this habit has been associated with STH infections (Wong *et al*., 1991; Geissler *et al*., 1998; Saathoff *et al*., 2002; Nchito *et al*., 2004). In a study, 46 % of geophagus school children carried out this activity at break hours in school (Geissler *et al*., 1998). Regardless of the season, by the mean parasite load for *Ascaris* and *Strongyloides*, children who practise geophagy are likely to ingest STH eggs/larvae; but infection may not be as frequent as when a higher parasite load is recorded (Steinbaum *et al*., 2016). In addition, some children that are not habitually geophagous, are equally at risk of infection because often time after play they were seen eating their snacks or other food items without strictly observing hand hygiene (personal observation). Further, in all the schools during break period, many of the boys often play in the open field (mainly football) without footwear so as to ease movement. Therefore, this risk behaviour further exposes these children to hookworm and *Strongyloides* infections.

The soil parasites recovered from designated points in the study locations may not be a complete reflection of the reported parasite profile as soil texture could affect egg recovery efficiency (Steinbaum *et al*., 2017). The flotation technique (sugar solution) used in this study is known to distort STH eggs and make microscopic identification difficult (Ayres & Mara, 1996). Also, identifying eggs in soil samples is challenging as soil contain different life stages of STH eggs. Worthy of note is that the rhabditiform larvae of *S. stercoralis* are morphologically similar to those of some free-living nematodes and it is possible that in some cases misidentification may have occurred. In future, molecular techniques should be used so that parasites are identified at species level as this could determine the extent to which these playgrounds are contaminated with human or zoonotic parasites.

Of the four soil parasites isolated (*Ascaris, Strongyloides*, hookworm and *Trichuris*), *Ascaris* was most dominant in the dry season while *Strongyloides* in the wet. The intensity of *Strongyloides* was higher in the wet than dry but not significant for other parasites. Clearly, schoolchildren in all the sampled areas across the state are substantially at risk of acquiring soil parasites; and we believe that the profile of parasites in Edo State public primary schools may be similar to other states in southern Nigeria as they have similar climatic conditions and possibly similar sanitary status. If sanitary conditions and the state of infrastructure remain unchanged, interrupting the cycle of infection would be daunting. To our knowledge, intervention programmes like preventive chemotherapy (WHO, 2006) within the context of mass drug administration has not been organised or implemented for Edo State and effectively in many parts of southern Nigeria. We cannot evidently provide reasons why such schemes have not been stepped down to heavily endemic regions like Edo State; but we sense a lack of political-will to push for this kind of programme. In any case, by this communication, we strongly advocate that relevant authorities and agencies should make efforts to implement this laudable project within the state and indeed in southern Nigeria as there are huge benefits (Bleakley, 2009). We also recommend that there should be significant improvement in the sanitary/water facilities in public primary schools including engaging in continuous education/enlightenment programme that strongly emphasise strict compliance to personal and environmental hygiene.

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Authors' contributions

CI designed the study; NPT, UCI, SAE, HOA, collected and processed samples; CI and AJO wrote the manuscript; while all read and approved the final version of the manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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Cormorant pellets as a tool for the knowledge of parasite-intermediate host associations and nematode diversity in the environment

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Summary

Anisakids are usually acquired through the diet. Cormorant pellets are useful to detect both parasite larval stages, and prey items which could act as intermediate hosts in the environment. The current study provides information about the feeding habits of both birds and mammals, and the diversity of parasites circulating in the environment. The objective of the study was to identify Anisakidae larvae and prey items in pellets from the Imperial shag *Phalacrocorax atriceps* and the Red-legged cormorant *P. gaimardi*, suggesting possible parasite–prey associations. A total of 92 *P. atriceps*' and 82 *P. gaimardi*'s pellets were collected from both Punta León, and Isla Elena bird colonies, respectively, during the period from 2006 to 2010. Pellets were preserved in ethanol and hard prey item remnants, and nematode larvae were studied using standard techniques. Prey item occurrence, nematode prevalence, and mean intensity were calculated. A correspondence analysis was performed to evaluate the larvae-prey association. *Contracaecum* spp., *Pseudoterranova* spp,, *Anisakis* spp., *Terranova* spp., and *Hysterothylacium* spp. third-stage larvae (L3) were identified in pellets. *Pseudoterranova* spp. and *Anisakis* spp. L3 predominated in the environment of Punta León, whereas *Contracaecum* spp. and *Hysterothylacium* spp. L3 predominated in the Puerto Deseado area. The highest larvae-prey association was that of *Contracaecum* spp. L3 with *Engraulis anchoita*, followed by with *Odontestes* sp. in *P. atriceps*' pellets. *Contracaecum* spp. L3 were significantly related to both sprats, *Sprattus fueguensis* and *Ramnogaster arcuatta*, in *P. gaimardi*'s pellets. It was verified that *E. anchovy* is the main gateway of *Contracaecum* spp. L3 in *P. atriceps*. *Odonthestes* sp. might act as an intermediate/paratenic host of *Contracaecum* spp. L3 in the area. Both sprats might play a role as intermediate/paratenic hosts of *C. australe*, being the main gateway into *P. gaimardi* in the area. Thus, pellet analysis can be postulated as a good tool for indicating parasite-host associations between anisakids, and the prey items which act as intermediate hosts.

Keywords: Anisakidae; pellets; *Phalacrocorax atriceps*; *Phalacrocorax gaimardi*, parasite-host association; Argentinean sea

Introduction

Most helminth parasites that occur in the marine environment have complex (indirect) life cycles. Among them, anisakid nematodes are important components in marine ecosystems (Rhode, 2005). Usually, the first step in the life cycle of the Anisakidae is an invertebrate (e.g. copepods) as first intermediate/paratenic host. Then, they frequently parasitize a fish as second intermediate/paratenic

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host, increasing the chances to reach their definitive host (DH), particularly piscivorous birds and mammals (Anderson, 2000; Rhode, 2005; Moravec, 2009). Therefore, the food web is the key to understanding the parasite community organization (Price, 1990; Anderson, 2000; Rhode, 2005).

The identification of host-parasite relationships and the role of each trophic item as intermediate, paratenic or definitive host are severely affected by difficulties associated in detecting endoparasites in each link of their life cycle. In such concern, the analysis of the stomach contents, pellets, and regurgitates of fish-eating birds, could be useful in detecting both larval parasites, and prey items that could act as intermediate/paratenic hosts in the environment.

The Imperial cormorant or shag *Phalacrocorax atriceps* (King, 1828), and the Red-legged cormorant *Phalacrocorax gaimardi* (Lesson & Garnot, 1828), are two of the five cormorant species nesting along the Argentinean coast. *Phalacrocorax atriceps* is distributed from Punta León, Chubut, to the Beagle Channel, Tierra del Fuego on the Argentinean coast (Harrison, 1983; Frere *et al*., 2005). *Phalacrocorax gaimardi* nests from Bahía Sanguinetto to Monte León, Santa Cruz Province (Frere *et al*., 2005). They are top predators in the marine food chain of the Patagonian coast, including mainly fish, and also mollusks or crustaceans in their diet, constituting an excellent model for the analysis of parasite-host interactions in the marine environment.

With the aim of determining if pellets of Phalacrocoracidae are indicators of larvae-intermediate/paratenic host association, the objectives of this study were to identify Anisakidae larvae and prey items found in pellets of both cormorants *P. atriceps* and *P. gaimardi*; to estimate interactions among them suggesting larvae-prey associations, and to suggest gateways of those anisakid species that are commonly found parasitizing both fish-eating birds (e.g. *Contracaecum* spp.).

Materials and Methods

From 2006 to 2010, a total of 92 pellets of *P. atriceps* were collected between bird nests at random in the Punta León cormorant colony, Chubut province, Argentina (43°05'S; 64°30'W) (Fig. 1), in two consecutive breading seasons: 47 pellets from December 2006 to January 2007, and 45 from December 2007 to January 2008. Later, 86 pellets of *P. gaimardi* were collected at random from November 2009 to January 2010 at the colony of Isla Elena, Ría Deseado, Santa Cruz province (47°45'S; 65°56'W) (Fig. 1). These 86 pellets are a subsample of a larger data set, which were used to describe *P. gaimardi* diet by Morgenthaler *et al*. 2016. In all birds, sampling was performed during the chick-rearing period from hatching up to the appearance of true feathers since this is the period of maximum activity for the food search (Yorio *et al*., 1998; Svagelj & Quintana, 2007).

Collected pellets were preserved in vials with 70 % ethanol, and once in the laboratory, they were disaggregated under a stereomicroscope. All hard prey remnants (e.g. otoliths, cephalopod beaks, etc), and nematode larvae were collected, cleared with lactophenol and studied under a light microscope (Garbin *et al*., 2007, 2008; 2011). Nematode identification was carried out following the appropriate taxonomic keys and bibliography (Hartwich, 1964, 1974; Fagerholm, 1990; Anderson, 2000). Parasite ecological indexes of prevalence (P), and mean intensity (MI) were calculated following Bush *et al*. (1997) only for larval stages. Prey items were identified by using reference collections, keys, and catalogues (Cousseau & Gru, 1982; Boschi *et al*., 1992; Gosztonyi & Kuba, 1996; Pineda *et al*., 1996; Volpedo & Echeverría, 2000). Prey occurrence was calculated for all items in pellets from both cormorant species.

A correspondence analysis (CA) was performed to evaluate the larvae-prey association (LPA) occurring in pellets from both fish-eating birds (Legendre & Legendre, 1998). This ordination technique allows the association of row (pellets) and column (species) frequencies in a contingency table. Prey item species with less than 5 % occurrence were excluded from the analysis since the rare taxa might introduce error and be placed at extreme ends of the first ordination axes relegating the major community trends to later axes (Gauch, 1982). We conducted all analyses with R 3.4.0 software (R Core Team 2017) using the vegan package (Oksanen *et al*., 2018).

Ethical Approval and/or Informed Consent

All pellets were collected without causing disturbance at both cormorant colonies of Punta León, Chubut, and Isla Elena, Santa Cruz, Argentina, with required permissions of the Dirección de Flora y Fauna Silvestre, Chubut, and the Dirección de Fauna Silvestre y Areas Protegidas, Santa Cruz, Argentina, respectively.

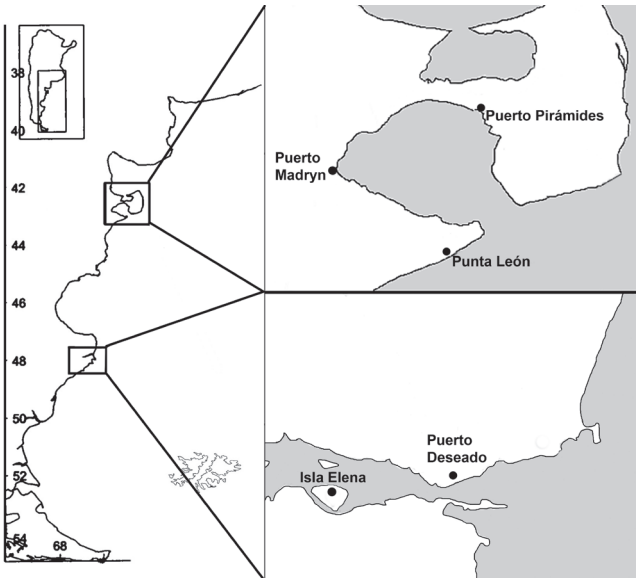

Fig. 1. Sampling sites of *Phalacrocorax atriceps* pellets at Punta León, Chubut coast, and *Phalacrocorax gaimardii* at Isla Elena, Ría Deseado, Santa Cruz Province, Argentina.

Results

From the analyzed *P. atriceps*'s pellets, 39 different prey items were identified, belonging to four different animal taxa: fish, mollusks, crustaceans, and polychaetes (Table 1). From *P. gaimardi*'s pellets, 10 different prey items were identified, belonging to the same four different animal taxa (Table 2).

Third-stage (L3), fourth-stage larvae (L4), and adults of five Anisakidae genera were identified in pellets from both cormorant species: *Contracaecum* Railliet & Henry, 1912, in both bird species; *Pseudoterranova* Mozgovoi, 1951, *Anisakis* Dujardin, 1845, *Terranova* Leiper & Atkinson, 1914, only in pellets of *P. atriceps*; and *Hysterothylacium* Ward & Magath, 1917, only in pellets of *P. gaimardi*. *Pseudoterranova* spp. L3 showed the highest prevalence (P=65.13) followed by *Anisakis* spp. L3 (P=43.66), *Contracaecum* spp. L3 (P=24.3), and *Terranova* spp. L3 (P=18.21) in *P. atriceps* pellets. The highest mean intensity was detected for *Pseudoterranova* spp. L3 (MI=4.32), followed by *Terranova* spp. L3 (MI=2.54), *Anisakis* spp. L3 (MI=1.92), and *Contracaecum* spp. L3 (MI=1.73). *Contracaecum* spp. L3 had the highest intensity and *Hysterothylacium* spp. L3 was the most prevalent anisakid in pellets of *P. gaimardi* (P=60.34, MI=3.25, and P=55.17, MI=4.56 respectively).

Only anisakid L3 were included in the CA analysis. The overall inertia was relatively low (2.893 out of 26) but still significant (χ^2 = 7111.2, N = 2458, P = <0.0001), indicating a weak association between parasites and prey items of *P. atriceps*. Moreover, the first two CA axes accounted for 37.86 % of the data. On *P. gaimardi*, the overall inertia was relatively higher (2.98 out of 7) and significant (χ^2 = 882.04, N = 296, P = <0.0001), indicating a stronger association between parasites and prey items. The first two CA axes accounted for 46.31 % of the data.

The highest significant larvae-prey association (LPA) in pellets of *P. atriceps* was revealed for *Contracaecum* spp. L3 with *Engraulis anchoita* Hubbs & Marini, 1935, followed by Afroditidae, *Odontesthes* sp., *Paralichthys* sp., Gammaridae sp., and Ostracoda (Fig. 2). *Pseudoterranova* spp. L3 showed a close relationship with Polyonidae, *Enteroctopus megalocyathus* (Gould, 1852), and *Octopus tehuelchus* d'Orbigny, 1834. *Anisakis* spp. L3 significantly associated with *Tegula* sp., followed by *Raneya brasiliensis* (Kaup, 1856), and *Percophis brasiliensis* Quoy & Gaimard, 1825. *Terranova* spp. L3 strongly associated with *Patagonotothen* sp., Eunicidae, *Triathalassothia argentina* (Berg, 1897), and *O. tehuelchus* (Fig. 2).

In *P. gaimardi*'s pellets, *Contracaecum* spp. L3 significantly associated with *Sprattus fueguensis* (Jenyns, 1842), followed by *Ramnogaster arcuatta* (Jenyns, 1842), *Loligo gahi* d'Orbigny, 1835, and *Patagonotothen* sp. *Hysterothylacium* spp. L3 associated with Nereididae, then with *S. fueguensis,* and *Odontesthes* sp. (Fig. 3).

Table 1. Occurrence of prey items in pellets of the Imperial shag *Phalacrocorax atriceps* from Punta León, Chubut province coast, Argentina.

Taxa	Species	Ocurrence (%)
Ophididae	*Raneya brasiliensis*	75.31
	Genypterus blacodes	1.23
Batrachoididae	*Triathalassothia argentina*	65.43
Clinidae	*Ribeiroclinus eigenmanni*	50.62
Nototheniidae	*Patagonotothen* sp.	19.75
Engraulidae	*Engraulis anchoita*	24.92
Agonidae	*Agonopsis chiloensis*	12.35
Pinguipedidae	*Pinguipes brasilianus*	10.28
	Pseudopercis sp.	12.35
Cheilodactylidae	*Nemadactylus bergi*	6.17
Serranidae	*Acanthistius brasilianus*	4.94
Zoarcidae	*Austrolycus laticinctus*	3.70
Paralichthyidae	*Paralichthys* sp.	4.02
	Xystreurys rasile	2.47
Percophidae	*Percophis brasiliensis*	2.43
Atherinopsidae	*Odontesthes* sp.	10.48
Merlucciidae	*Merluccius hubbsi*	5.23
Triglidae	*Prionotus* sp.	1.28
Myxinidae	*Myxine* sp.	1.25
Rajidae	*Raja* sp.	1.2
Octopodidae	*Enteroctopus megalocyathus*	22.22
	Octopus tehuelchus	19.75
Bivalvia	Heterodonta	9.88
Prosobranchia	*Tegula sp.*	8.64
Ostracoda	Ostracoda	8.64
Amphipoda	Gammaridae	2.47
Anomura	*Pachicheles chubutensis*	2.47
Caridea	*Atlantopandalus* sp.	4.94
	Nauticaris sp.	1.29
	Chorismus sp.	1.26
	Campylonotus sp.	1.23
	Betaeus sp.	1.21
	Pterygosquilla sp.	1.19
Brachiura	*Coenophthalmus tridentatus*	4.93
Solenoceridae	*Pleoticus muelleri*	1.23
Polychaeta	*Eunice* sp.	12.35
	Polyonidae	6.17
	Aphrodita sp.	3.70

Discussion

The Imperial shag in Punta León colony showed a preferably piscivorous diet with common prey items being *R. brasiliensis, T. argentina* and *Ribeiroclinus eigenmanni* (Jordan, 1888) (Malacalza *et al*., 1994; Punta *et al*., 2003). Fish such as *S. fueguensis* and

Table 2. Occurrence of prey items in pellets of the Red-legged cormorant *Phalacrocorax gaimardi* from Isla Elena, Ría Deseado, Santa Cruz province coast, Argentina.

Taxa	Species	Ocurrence (%)
Clupeidae	*Sprattus fueguensis*	32.86
	Ramnogster arcuata	14.29
Nototheniidae	*Patagonotothen sp.*	2.86
Pinguipedidae	*Pinguipes brasilianus*	1.43
Atherinopsidae	*Odontesthes sp.*	10.68
Zoarcidae	Zoarcid	1.43
Loliginidae	*Loligo gahi*	8.57
	Loligo sp.	12.86
Crustacea	*Eurypodius latreilli*	4.29
Nereididae	Nereidid	4.23

R. arcuatta were also the most frequent items in the Red-legged cormorant diet (Morgenthaler *et al*., 2016). Recorded anisakid prevalences suggested that *Pseudoterranova* spp. L3. and *Anisakis* spp. L3 predominated in the environment over the other two anisakid genera in the Punta León sea area, Chubut coast, whereas *Contracaecum* spp. L3 and *Hysterothylacium* spp. L3 predominated in the environment of Puerto Deseado, Santa Cruz coast. Thus, this study showed that pellets might serve to indicate the general diversity of the environment.

Contracaecum is the only genus of Anisakidae in this study that matures to adults in birds. The most significant LPA found in *P. atriceps* pellets was that of *Contracaecum* spp. L3 with *E. anchoita*. Previously, Diaz (2006), and Garbin *et al*. (2007) had suggested that *E. anchoita* might act as a transmitter of *Contracaecum pelagicum* L3 to the Magellanic Penguin *Spheniscus magellanicus* (Forster, 1781) and *P. atriceps*. Later, Garbin *et al*. (2013) proved this presumption analyzing phylogenetically through molecular markers (mtDNA cox2, rrnS, ITS-1, ITS-2 genes) specimens of *Contracaecum* spp. L3 from *E. anchoita*, and *C. pelagicum* of both sympatric fish-eating birds *P. atriceps* and *S. magellanicus* from Península Valdés (Garbin *et al*., 2013), the same area where the present pellets were collected. From these results, we can verify that *E. anchovy* is the main gateway of *Contracaecum* spp. L3 into *P. atriceps*. Therefore, the analysis of pellets was efficient to suggest possible parasite-host associations.

In the present analysis, *Contracaecum* spp. L3 also showed an association with *Odontesthes* sp. Carballo *et al*. (2011) reported *Contracaecum* L3 parasitizing the silversides *Odontesthes smitti* (Lahille, 1929), and *Odontesthes nigricans* (Richardson, 1848) in Península Valdés coast, Argentinean Sea. Present results reinforced the idea that *Odonthestes* sp. also should act as an intermediate/paratenic host of *Contracaecum* spp. L3 in the area. Phylogenetic molecular studies should be carried out to confirm this LPA hypothesis (Garbin *et al*. 2013).

Another significant LPA found was that between *Contracaecum* spp. L3 and the polychaete *Aphrodita* sp. Some records of *Contracaecum* parasitizing polychaetes exist but not in the Aphroditidae to date (Peoples, 2013).

Lower LPA were those between *Contracaecum* spp. L3 and *Paralichthys* sp., Gammaridae and Ostracoda. Incorvaia & Díaz de Astarloa (1998) also found *Contracaecum* L3 in *Paralichthys orbignyanus* (Valenciennes, 1839), and *Paralichthys patagonicus* Jordan, 1889, from the Argentine sea. Some authors have suggested gammarid amphipods and ostracods as intermediate/paratenic hosts of *Contracaecum* spp. larvae (Bartlett, 1996; Anderson, 2000; Moravec, 2009). Related to this, some ostracod species are prey items of *E. anchoita* and *Paralichthys* sp. (Capitanio *et al*., 2005; Ide *et al*., 2006). Therefore, it is possible to suggest that these arthropods are involved in some stage of the *Contracaecum* life cycle in the study area.

In this study, *Pseudoterranova* spp. L3 were the most abundant anisakid found showing the highest association with Polyonidae. McClelland *et al*. (1990) found *Pseudoterranova* L3 in polychaetes after they ingested copepods experimentally. Also, Martell & McClelland (1995) pointed out polychaetes as transmitters of *Pseudoterranova* L3. Associations of *Pseudoterranova* spp. L3 with *Enteroctopus* sp., and *Octopus* sp. are strange since there are no records of this anisakid parasitizing those cephalopods. *Terranova* spp. L3 strongly associated with *Patagonotothen* sp, Eunicidae,

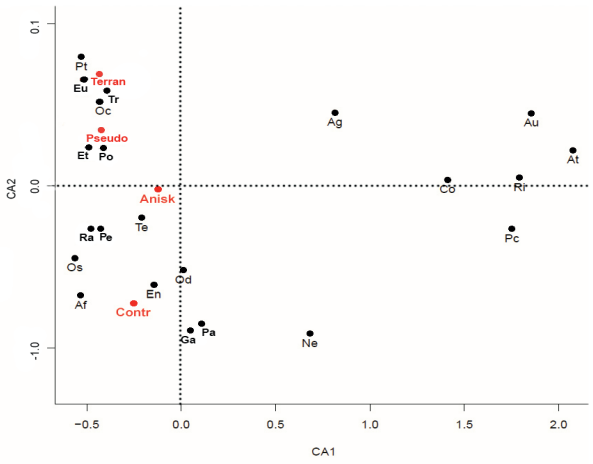

Fig. 2. Results of Correspondence Analysis (CA) revealing the larvae-prey associations in pellets of *Phalacrocorax atriceps* from Punta León, Chubut coast, Argentina. Contr: *Contracaecum* sp. L3; Anisk: *Anisakis* sp. L3; Pseud: *Pseudoterranova* sp. L3; Terran: *Terranova* sp. L3; En: *Engraulis anchoita*; Od: *Odontesthes* sp.; Ra: *Raneya brasiliensis*; Tr: *Triathalassothia argentina*; Ri: *Ribeiroclinus eigenmanni*; Pt: *Patagonotothen* sp.; Ag: *Agonopsis chiloensis*; Au: *Austrolycus laticinctus*; Pa: *Paralichthys* sp.; Pe: *Percophis brasiliensis*; Et: *Enteroctopus megalocyathus*; Oc: *Octopus tehuelchus*; Os: Ostracoda; Ga: Gammaridae; At: *Atlantopandalus* sp.; Co: *Coenophthalmus tridentatus*; Eu: *Eunice* sp.; Po: Polynoidae; Af: *Aphrodita* sp.

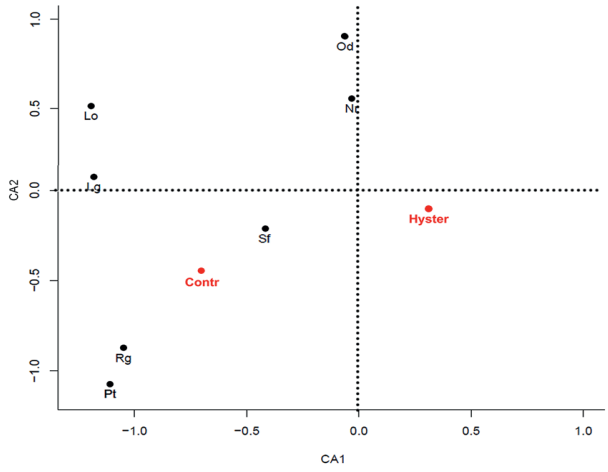

Fig. 3. Results of Correspondence Analysis (CA) revealing the parasite-pray associations in pellets of *Phalacrocorax gaimardii* from Isla Elena, Cruz Province, Argentina. Contr: *Contracaecum* sp. L3; Hyster: *Hysterothylacium* sp. L3; Sp: *Sprattus fueguensis*; Rg; *Ramnogster arcuata*; Od: *Odontesthes* sp.; Pt: *Patagonotothen* sp.; Lg: *Loligo gahi*; Lo: *Loligo* sp.; Ne: Nereididae.

and *T. argentina*, and *Octopus* sp. However, there are no records of this anisakid genus parasitizing any of the latter prey items up to date. Despite *Anisakis* spp. L3 closely associated with *Tegula* sp., it is not possible to speculate a parasite-host association because there are no records of gastropods as intermediate/paratenic host of anisakid species. None of the mentioned genera are parasites of birds.

The analyses carried out on *P. gaimardi*'s pellets from the Ría Deseado showed the two highest LPA of *Contracaecum* spp. L3 with both sprat species *S. fueguensis* and *R. arcuata*. Other studies have confirmed the parasitism of *Contracaecum* in the genus *Sprattus* (Skrzypczak & Rolbiecki, 2015; Zuo *et al*., 2016). In addition, *Contracaecum australe* Garbin *et al*., 2011, was reported previously parasitizing *P. gaimardi* in the same area (Garbin *et al*. 2014). Therefore, both sprats *S. fueguensis* and *R. arcuata* might play a role as intermediate/paratenic hosts of *C. australe*, being the main gateway to *P. gaimardi* in the study area. As mentioned before, phylogenetic molecular studies should be carried out on these nematodes (Garbin *et al*. 2013).

Also, significant LPA between *Hysterothylacium* spp. L3 with Nereididae, *S. fueguensis* and *Odontesthes* sp. were observed in *P. gaimardi*'s pellets. No records of *Hysterothylacium* parasitizing this polychaete are available. However, there are some records on *Hysterothylacium aduncum* (Rudolphi, 1802) isolated from *Sprattus sprattus* (Linnaeus, 1758) in different geographical areas in the Northeastern Atlantic and Southern Baltic Sea (Klimpel *et al*. 2007; Skrzypczak & Rolbiecki, 2015). In addition, *Odontesthes bonariensis* (Valenciennes, 1835) is parasitized by *Hysterothylacium* sp. larvae from two Argentinean lagoons (Drago, 2012). Thus, it is possible to suggest *S. fueguensis* and *Odontesthes* sp. as first intermediate hosts of *Hysterothylacium* spp. L3 since the adult nematodes parasitize other teleost fish.

In this study, *Contracaecum* also showed LPA with the Patagonian squid *L. gahi* in *P. gaimardi*. Some cephalopods have been recorded to be parasitized by *Contracaecum* L3 as paratenic -transport- hosts (Shukhgalter & Nigmatullin, 2001; Salati *et al*., 2013). However, only *Loligo forbesi* (Steenstrup, 1856) was shown to be infected with *Contacaecum* L3 (Smith, 1984). Not only surveys on this squid are needed but also molecular studies on *Contacaecum* L3 must be carried out.

According to the present results it is possible to postulate pellets as good tools to indicate parasite-host associations between anisakids and the prey items which act as intermediate/paratenic hosts. These kind of studies also provide information about the feeding habits of both birds and mammals, and about the diversity of parasites circulating in the environment.

Conflict of Interest

Authors state no conflict of interest.

Acknowledgments

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Changes in haematological parameters in wild ruminants experimentally infected with *Haemonchus contortus*

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Article info

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Summary

Our study describes changes in haematological parameters in wild ruminants with parasitic infection. Six European mouflons (*Ovis musimon*), six fallow deer (*Dama dama*) and six roe deer (*Capreolus capreolus*) were experimentally infected with the resistant strain of the model parasite 8000 L3 *Haemonchus contortus*. The blood samples were collected on Day 0, 16, 37, 58, 77, and 99 of the experiment. Mild anaemia was observed in mouflons and roe deer while red blood cells increased in red blood counts (total erythrocytes, haematocrit and haemoglobin). As for the white blood cells count, leucopenia with neutrophilia and lymphopenia was recorded in mouflons, in the fallow deer and roe deer leucocytosis with neutropenia and lymphocytosis were observed. Changes in the dynamics of haematological parameters were statistically insignificant.
Keywords: mouflons; fallow deer; roe deer; *Haemonchus contortus*; haematological parameters

Introduction

Parasitic infections in wild ruminants may cause high morbidity, and in the case of heavy infections, immunosuppression and poor body mass condition, they may even lead to death of the animal. The negative effect of parasitic infection depends on the host's nutritional status, gender – males have a weaker immune system than females, age – young and elderly individuals are more susceptible to infection than adults (Jégo *et al*., 2014). Most studies have focused on the impact of parasites on the nutritional status of wild ruminants, but this has been confirmed as a nonspecific and slow indicator of parasitic infection (Irvine *et al*., 2006; Jégo *et al*., 2014). More specific and faster diagnostic indicator of the presence of parasitic infection may be host haematology parameters (Beldomenico & Begon, 2010). Changes in the health status of wild ruminants may also be reflected in haematological parameters (Ciberej *et al*., 2007; Mašek *et al*., 2009). Monitoring their dynamics contributes greatly to diagnosis, differential diagnosis, assessment of the effect of therapy as well as prediction of a course of diseases in animals. However, blood analysis may also indicate an accurate status of nutrition, trauma, and environmental stressors (Peréz *et al*., 2003). *Haemonchus contortus* is one of the hematophagous species that can cause haemorrhagic anaemia, especially in young and immunosuppressed animals. If the infection persists for a long time (chronic blood loss), it can lead to a rapid depletion of the erytrocyte stocks in the spleen and bone marrow and anaemia begins to develop, characterized by a decrease in all red blood component parameters (Doubek *et al*., 2003; Kolodziej-Sobocińska *et al*., 2016). Changes observed in the white blood cells in wild ruminants may result from an organism's response to stress (stress leukocytosis) and inflammation as a result of parasitic and allergic reactions (Ciberej *et al*., 2007). The values of haematological parameters of both the red and white blood cells are dependent on many factors such as breed,

* – corresponding author

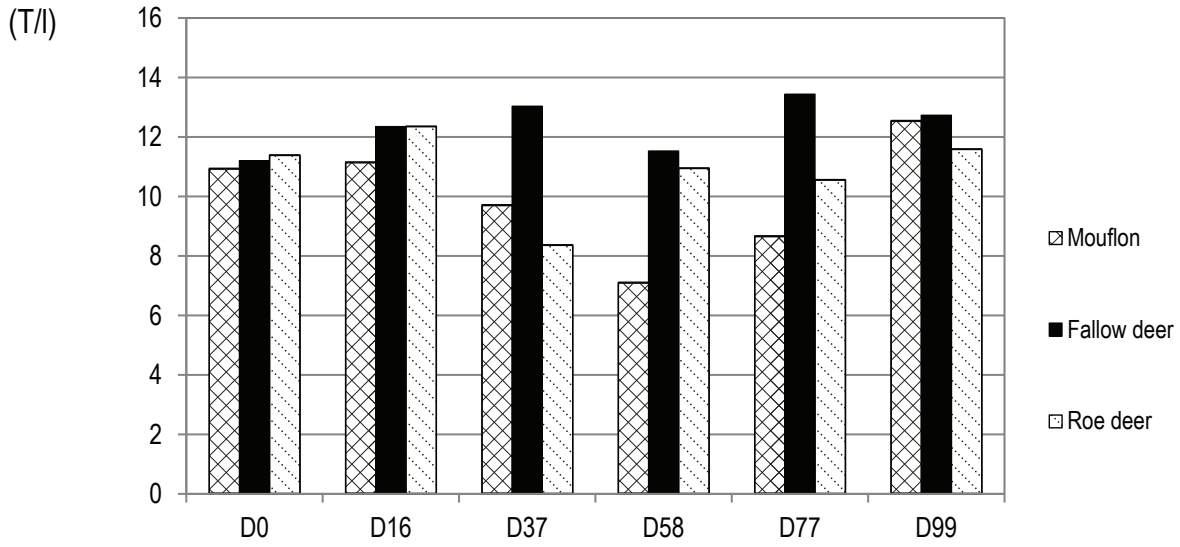

Fig. 1. Results of total number of erythrocytes (T/l) in three species of wild ruminants infected with resistant (MHco4) strain of *H. contortus*. Day (D) 0 (day of infection), D16, D37, D58, D77 (days post infection), D77 (day of treatment), D99 (D21 after treatment).

age, sex, pregnancy and season (Küker *et al*., 2015).

Vengušt *et al*. (2006) and Casas-Diaz *et al*. (2008) also reported that the method of capture and fixation of wild ruminants can greatly affect the blood parameter values. Vengušt *et al*. (2006) in fallow deer recorded higher values in total erythrocyte count, haemoglobin and haematocrit in subjects immobilized by chemical methods compared to animals immobilized by physical methods.

The aim of this study was to describe changes in total number of erythrocytes and leukocytes, haematocrit and haemoglobin and leukogram values (differential cell blood counts) in wild ruminants (mouflons, fallow deer, roe deer) experimentally infected with the model parasite *H. contortus*.

Material and Methods

Three species of wild ruminants – six European mouflons (*Ovis musimon*), six fallow deer (*Dama dama*) and six roe deer (*Capreolus capreolus*) with an average age of 1.5 years were experimentally infected with the model parasite *H. contortus*. Each animal was housed individually, fed with meadow hay and a commercial

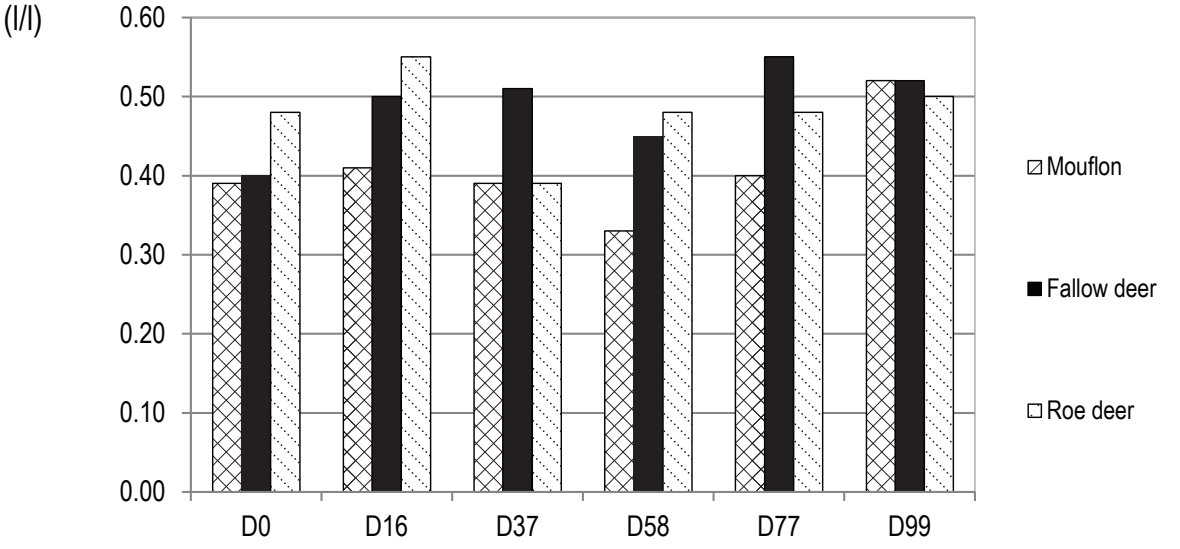

Fig. 2 Results of haematocrit values (l/l) in three species of wild ruminants infected with resistant (MHco4) strain of *H. contortus*. Day (D) 0 (day of infection), D16, D37, D58, D77 (days post infection), D77 (day of treatment), D99 (D21 after treatment).

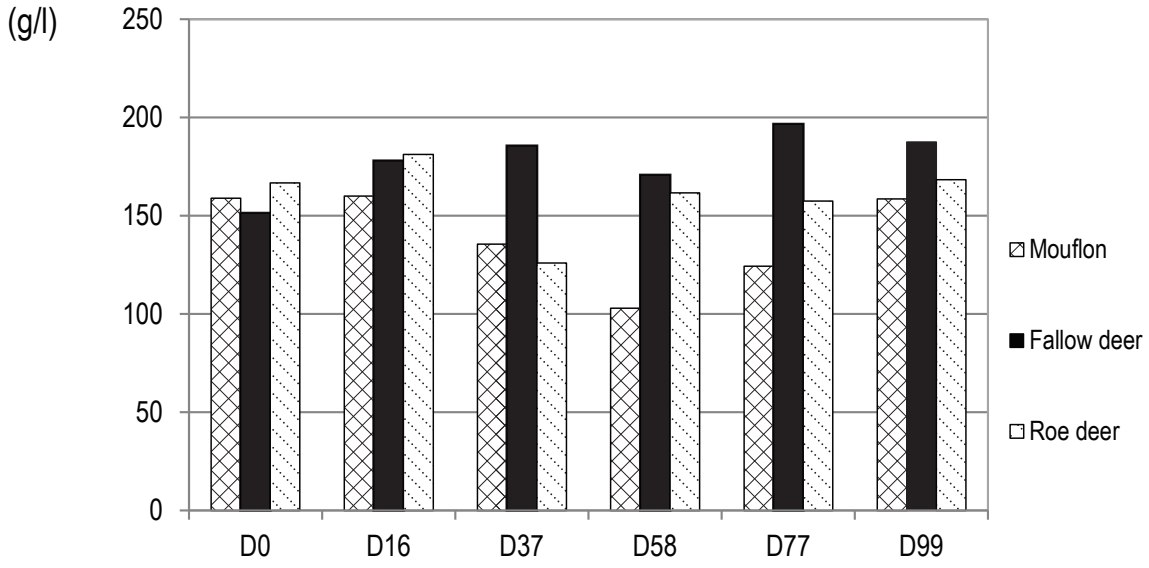

Fig. 3 Results of haemoglobin values (g/l) in three species of wild ruminants infected with resistant (MHco4) strain of *H. contortus*. Day (D) 0 (day of infection), D16, D37, D58, D77 (days post infection), D77 (day of treatment), D99 (D21 after treatment).

concentrate with free access to water, mineral and salt licks.

All animals were treated *per os* with albendazole (10 mg / kg body weight, ALDIFAL 2.5 % susp. a.u.v., MEVAK, Slovakia) prior to the experiment for the purpose of utilising parasite free animals. Faecal samples from all experimental animals were examined and negative coprological findings were confirmed before the experiment. After a week, each animal was infected *per os* with 8000 L3 larvae of isolate of *H. contortus* (MHco4). MHco4 is a multi-resistant (ivermectin, BZ, rafoxanide, closantel) field isolate from South Africa (Van Wyk & Malan, 1988). The dynamics of haematological parameter values in all animals were monitored for 99 days. Blood samplings were performed on day (D) 0, day of infection, D16, D37, D58 and D77 days post infection (p.i.). On D77 p.i., levamizole was orally administered to all experimental animals with Ripercol Drench (Elanco, Germany) at a dose of 2 ml / 10 kg body weight. Blood was again collected on D99 p.i. of the experiment (D21 after treatment). Each animal was immobilized prior to the blood collection by applying Hellabrun mixture (125 mg xylazine

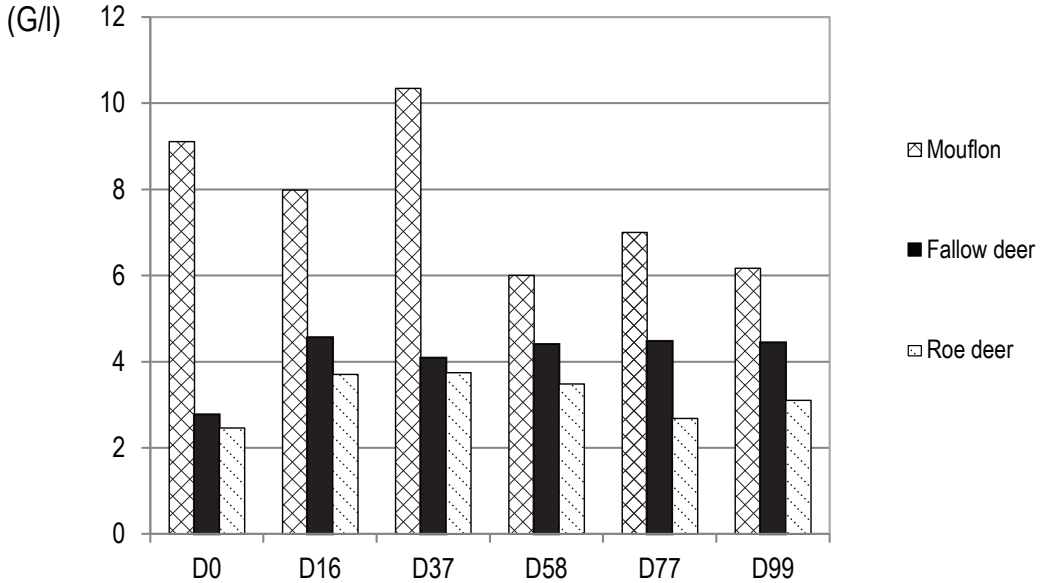

Fig. 4 Results of the total leucocyte count (G/l) in three species of wild ruminants infected with resistant (MHco4) strain of *H. contortus*. Day (D) 0 (day of infection), D16, D37, D58, D77 (days post infection), D77 (day of treatment), D99 (D21 after treatment).

Table 1. Leucogram results - granulocytic line in three species of wild ruminants infected with resistant (MHco4) strain of *H. contortus* (%).

	Day of blood collection	Mouflon *(Ovis musimon)*	Roe deer *(Dama dama)*	Fallow deer *(Capreolus capreolus)*
			Average ± SD	
Neutrophilic granulocytes (%)	D0 - infection	33.80 ± 1.51	45.60 ± 0.63	93.20 ± 0.35
	D16	21.80 ± 5.58	34.13 ± 1.59	85.33 ± 1.11
	D37	51.17 ± 2.01	33.20 ± 2.46	83.90 ± 0.53
	D58	50.00 ± 2.16	36.60 ± 0.88	86.10 ± 0.77
	D77 + treatment	54.67 ± 5.43	32.46 ± 6.64	88.20 ± 1.15
	D99 - D21 post treatment	41.00 ± 4.32	39.20 ± 0.65	87.13 ± 0.12
Eosinophilic granulocytes (%)	D0 - infection	1.80 ± 0.43	3.16 ± 0.12	3.36 ± 1.23
	D16	2.10 ± 0.16	3.13 ± 0.26	7.26 ± 0.60
	D37	1.67 ± 0.47	2.46 ± 0.36	8.30 ± 0.43
	D58	2.33 ± 0.47	2.33 ± 0.47	8.63 ± 0.52
	D77 + treatment	2.00 ± 0.0	1.97 ± 0.04	9.00 ± 1.48
	D99 - D21 post treatment	3.24 ± 1.43	1.96 ± 0.04	8.36 ± 0.57
Basophilic granulocytes (%)	D0 - infection	0.93 ± 0.73	4.80 ± 0.24	1.36 ± 0.33
	D16	0.63 ± 0.44	3.40 ± 0.29	4.63 ± 0.52
	D37	0.33 ± 0.47	4.60 ± 0.08	4.10 ± 1.42
	D58	0.33 ± 0.47	2.63 ± 0.44	2.56 ± 0.70
	D77 + treatment	0.67 ± 0.47	4.90 ± 0.08	1.36 ± 0.46
	D99 - D21 post treatment	2.10 ± 0.78	4.33 ± 0.26	2.16 ± 0.52

hydrochloride 100 mg ketamine hydrochloride / 1 ml) at the following doses: mouflon 1.5 – 2.0 ml, fallow deer 2.0 – 2.5 ml and roe deer 0.2 – 0.3 ml. Blood was collected from the jugular vein into an ethylene-diamine-tetraacetic acid anticoagulant tube (EDTA) and processed on an IDEXX ProCyte DxTH (IDEXX Laboratory, Cymedica). The mean and ± statistical deviations were calculated using the Excel 2010 statistical program (Microsoft Int.).

Individual counts of eggs per gram (EPG) for each experimental animal were determined by a modified McMaster technique with a sensitivity of 50 EPG (Coles *et al*., 1992; 2004). Three grams of faeces from each animal were mixed with 42 ml of water and passed through a sieve. The filtrate was centrifuged at 605 g for 2 min. The sediment was mixed with a sugar solution with specific gravity 1.28 and centrifuged under the same conditions. One millilitre of the mixture was then transferred to McMaster chambers.

Tukey-Kramer test (one-way ANOVA) was used to evaluate the haematological parameters. A significance level of less than 5 % ($P<0.05$) was considered statistically significant.

Ethical Approval and/or Informed Consent

Animal use and study design were approved by the Ethics Committee of the Institute of Parasitology of the Slovak Academy of Sciences in accordance with the national legislation in Slovakia − Animal Welfare Act No. 3/2009.

Results

Mouflons

In the parameters of the red blood count (total erythrocytes, haematocrit and haemoglobin values), mild anaemia was noted on D37, D58 and D77 p.i. (Figs. 1, 2, 3), with the most pronounced decrease in these parameters on D58 p.i. Mild anaemia was caused due to erythrocyte loss during *H. contortus* infection. Considering the day of infection, leukocytopenia with neutrophilia and lymphopenia was detected in the total leukocyte count (Fig. 4). The most pronounced leukopenia was determined on D58 and D77 p.i. and neutrophilia and lymphopenia on D37 p.i. (Table 1, 2, Fig. 4). Changes in these values persisted until the end of the experiment. Values recorded in total leukocytes, neutrophil granulocytes and lymphocytes indicate a high level of parasitic infection. Simultaneously, a low level of posthemorrhagic anaemia was confirmed with the presence of acute local mucosal inflammation. Parasitic infection may be accompanied by eosinophilia. Changes in the values of eosinophil granulocytes were caused by *H.concortus* infection. Similarly, in our results eosinophilia was observed throughout the

Table 2. Leucogram results - Agranulocytic line in mouflons (*Ovis musimon*), roe deer (*Capreolus capreolus*) and fallow deer (*Dama dama*) during the experimental infection with resistant (MHco4) strain of *H. contortus* (%).

	Day of blood collection	Mouflon *(Ovis musimon)*	Roe deer *(Dama dama)*	Fallow deer *(Capreolus capreolus)*
			Average ± SD	
Lymphocytes (%)	D0 - infection	61.53 ± 1.26	39.80 ± 0.53	2.06 ± 1.06
	D16	71.90 ± 4.30	53.33 ± 2.51	2.56 ± 1.14
	D37	42.70 ± 2.65	54.03 ± 2.08	3.70 ± 1.10
	D58	45.33 ± 2.35	54.36 ± 0.73	2.70 ± 0.74
	D77 + treatment	40.33 ± 4.02	56.63 ± 6.50	1.43 ± 0.49
	D99 - D21post treatment	50.53 ± 2.60	50.43 ± 0.75	2.33 ± 1.13
Monocytes (%)	D0 - infection	1.60 ± 0.50	6.63 ± 0.89	0±0
	D16	3.57 ± 1.30	6.00 ± 0.81	0±0
	D37	3.47 ± 0.44	5.70 ± 0.53	0±0
	D58	2.00 ± 0.81	4.06 ± 0.17	0±0
	D77 + treatment	2.66 ± 1.24	4.03 ± 0.12	0±0
	D99 - D21post treatment	2.77 ± 0.20	4.06 ± 0.16	0±0

experiment. Monocytosis was recorded throughout the experiment with maximal value on D16 p.i. (Table 2), what indicated an acute inflammatory process due to parasitic infection.

Fallow deer

The increase in red blood counts was determined on all sampling days, with a maximum on D77 p.i. (Figs. 1, 2, 3). Leukocytosis with neutropenia and lymphocytosis was recorded in on all sampling days (Table 1, 2, Fig. 4). The eosinophil granulocyte values decreased throughout the experiment. In fallow deer group, basophilia was not detected (Table 1). Monocyte levels decreased on D58 p.i. (Table 2).

Following the treatment with levamisole on D99 p.i., red blood cell maximal values were recorded in all experimental species what could be related to the immunostimulatory effect of levamisole (Figs. 1, 2, 3). The changes recorded in the dynamics of the haematological parameters of the red and white blood line during parasitic infection with resistant strain (MHCo4) of *H. contortus* were statistically insignificant in all experimental species of wild ruminants. Changes recorded in red and white blood counts were associated with the rate of parasitic infection, expressed as eggs per gram of faeces – EPG (Table 3).

Roe deer

Mild anaemia was recorded on D37, D58 and D77 p.i. with the most pronounced decrease on D37 p.i. in comparison with the day of infection (Figs. 1, 2, 3). Leukocytosis with neutropenia and lymphocytosis was determined in total leukocyte count on all sampling days (Table 1, 2, Fig. 4). Changes in the mentioned parameters in roe deer could be caused by a lower intensity of parasitic infec-

Table 3. Mean egg count±standard deviation for the three species of wild ruminants – European mouflon (*Ovis musimon*), roe deer (*Capreolus capreolus*) and fallow deer (*Dama dama*) during the experimental infection with resistant (MHco4) strain of *H. contortus*.

	EPG D16	EPG D37	EPG D58	EPG D77	EPG D99
Mouflon n=3	0.0 ± 0.0	12 250 ± 105.48	25 400 ± 432.00	19 850 ± 550.00	0 ± 0
Fallow deer n=3	100 ± 60.28	150 ± 55.20	150 ± 55.54	0 ± 0	0 ± 0
Roe deer n=3	0.0 ± 0.0	50 ± 47.25	150 ± 57.35	0 ± 0	0 ± 0

EPG, eggs per gramme, number of *H. contortus* eggs in 1 gramme of faeces

tion without an acute abomasal mucosal inflammation (Table 3). Eosinophilia was noted in the roe deer throughout the experiment with a maximum value on D77 p.i. in comparison with the day of infection (Table 1). It can be assumed that the recorded basophilia followed by eosinophilia may have resulted from an allergic reaction to the immune mobilizing pharmaceutical metabolites.

Discussion

It is well known that many species of gastrointestinal nematodes are common to domestic and wild ruminants due to their frequent common grazing on pastures. Little is known about the haematological profile of wild ruminants infected with *H. contortus*, one of the most pathogenic hematophagous gastrointestinal nematodes occurring in domestic and wild ruminants. Ferté *et al*. (2000) summarised and compared the prevalence of *H. contortus* in three species of wild ruminants (red, roe, and fallow deer) from surveys carried out across Europe. A total of 36 surveys in 17 European countries recorded the incidence of *H. contortus* in roe deer, with prevalence ranging from 0.3 to 85 %. *H. contortus* was found in red deer in nine countries, with prevalence between 5 and 25 %, and in fallow deer in four countries (4 – 7 %) (Ferté *et al*., 2000). Cerutti *et al*. (2010) confirmed the low host specificity of *H. contortus* by finding it in common populations of roe deer, chamois, alpine ibex, and domestic goats and sheep in various alpine areas. Mašek *et al*. (2009) noted the differences in blood analyses under physiological conditions between mouflon and sheep, Ciberej *et al*. (2007) described haematological profile of mouflons; however no data compares changes in blood parameters between three different species of wild ruminants during infection with hematophagous parasites.

The first sign of the *H. contortus* infection is a decrease in values in red blood cell count. The red and white blood count may interpret differences related to nutritional status, disease condition and environmental impact. The progression and prognosis of the disease depend on several factors: parasitic infection intensity, age, breeds and host health. The main pathogenic mechanism of *H. contortus* parasite lies in a direct damage to the gastric mucosa and subsequent blood sucking (Angulo-Cubillan *et al*., 2007). Haemorrhagic anaemia is a result of a strong infection characterized by pale gingiva and conjunctival sac. There is also a decrease in total erythrocyte count, hypoalbuminemia and total protein in haematological parameters (Alvarez *et al*., 2000). Lavin *et al*. (1997) described similar changes in red blood counts in wild Spanish goats with present *H. contortus* in the abomasum. Macrocytic hypochromic anaemia, anisocytosis, poikilocytosis and occasionally the Howell-Jolly body were diagnosed in the erythrocytes. The authors assumed that anaemia was caused by acute bleeding. Kolodziej-Sobocińska *et al*. (2016) reported similar changes in European bison infected with *Ashworthius siedmi* (*Trichostrongylidae*). Erythrocyte loss can be balanced by increased reticulocyte production, but if losses are too high anaemia occurs. Our results indicated that mild anaemia occurred only in mouflons and roe deer. The level of decline in red blood cell parameters correlated with increasing intensity of parasitic infection expressed in EPG values. After the deworming, the red blood cell values were adjusted, confirming that irreversible changes did not occur in the early therapy of the disease in the blood and the organism was able to regenerate relatively quickly. Post-haemorrhagic anaemia in experimental wild ruminants was reversible as erythrocyte reserves in the bone marrow and spleen were sufficient to compensate for their loss in peripheral blood. Higher values of red blood cell parameters were noted in fallow deer, probably due to a lower sensitivity to infection with *H. contortus*. It can be assumed that the indicated parasitic infection rate in fallow deer was not high enough and long-term to cause a decrease in these values, respectively. Parasitic infection of animals in poor nutritional and condition status might also be of higher intensity because parasites would encounter less opposition to their survival. It is generally acknowledged that discrepancy in susceptibility is one reason why parasites tend to be concentrated in individual host (Beldomenico & Begon, 2009). Peinado *et al*. (1999) confirmed that the physiological values of total erythrocytes, haematocrit and haemoglobin are higher in young fallow deer. Similarly, Vengušt *et al*. (2006) reported that the total erythrocyte count, haemoglobin and haematocrit are also affected by the trapping method. Haematological values in fallow deer immobilized by chemical methods were higher than in fallow deer immobilized by physical methods.

The effect of catecholamine release is expected to cause spleen contraction and subsequent release of erythrocytes into peripheral blood. The results of haematological parameters may also be influenced by season, age, gender and pregnancy (Küker *et al*., 2015). In mouflons, leukopenia was recorded with neutrophilia and lymphopenia, eosinophilia and monocytosis, indicative of intense haemonchosis accompanied by post-haemorrhagic anaemia and suspected local acute mucosa inflammation in abomasum. Kraft & Dürr (2005) described pathological neutrophils with a regenerative left shift even during posthemorrhagic anaemia. In the fallow deer and roe deer, leukocytosis with neutropenia and lymphocytosis was recorded. Haematological parameters for roe deer and fallow deer did not indicate an acute inflammation associated with low parasitic infection intensity. Distinct eosinophilia accompanied by basophilia was recorded in the roe deer after D16 p.i. It can be assumed that an allergic reaction of the organism to the interaction of the parasitic infection and the immobilizing agent used has occurred. In comparison to the study of Peinado *et al*. (1999) our eosinophil granulocyte baseline values in roe deer group were increased. Eosinophilia can be caused by the chemicals used to immobilize experimentally infected wild ruminants. Similar changes in the white blood count in wild ruminants which can result from an organism's response to local or total inflammation as a result of parasitic and allergic reactions were reported by Ciberej *et al*. (2007).

Conclusion

Our results indicated that changes in both red and white blood counts observed in infected wild ruminants were temporary. The degree of damage caused by *H. contortus* was dependent on the number of parasites present and wild ruminant's species sensitivity. Changes in the red blood cell indicated a development of haemorrhagic anaemia. Changes in the white blood count could be attributed to the intensity of infection and the animal's response to pathogenesis of *H. contortus* and its subsequent local inflammatory response with the presence of haemorrhages. These changes would be more pronounced in case of a severe parasitic infection, poor body condition or in immunosuppressed hosts.

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Conflict of Interest

Authors state no conflict of interest.

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Studies on gastrointestinal helminth of three Lacertid Lizard species, *Podarcis muralis, Podarcis siculus* and *Ophisops elegans* (Sauria: Lacertidae) from Bursa, North-Western Turkey

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Summary

A total of 80 specimens of three species of lacertid *Podarcis muralis* (39), *Podarcis siculus* (18) and *Ophisops elegans* (23) from Bursa were examined for helminths. One species of Digenea, *Plagiorchis elegans*, 1 species of Cestoda, *Mesocestoides* sp. (tetrathyridium); and 3 species of Nematoda, *Skrjabinodon medinae, Spauligodon saxicolae* and *Skrjabinelazia hoffmanni* were found. The helminths reported in this study are generalist helminths that infect a number of lizards.
Keywords: *Podarcis muralis; Podarcis siculus*; *Ophisops elegans*; Digenea; Cestoda; Nematoda

Introduction

Common Wall Lizard, *Podarcis muralis* (Laurenti, 1768) inhabits dry, sunny, rocky places, sometimes sparsely wooded areas; seen on garden walls and ruins. This species known from Middle and South Europe and Turkey; with a vertical distribution up to 2000 m. Istanbul Wall Lizard, *Podarcis siculus* (Rafinesque-Schmaltz, 1810) prefers rocky-stony places and rough stone walls, seen on garden walls or in cemeteries. Its range includes South Europe and North- west Turkey, also an isolated colony in Philadelphia (USA); with a vertical distribution up to 1800 m. A single subspecies *P. s. hieroglyphica* Berthold, 1842 lives in Turkey, in urban Istanbul and Bursa and some islands in the Sea of Marmara (Ugurtas *et al*. 2000). Snake – eyed Lizard, *Ophisops elegans* Menetries, 1832 is a ground-dwelling species usually inhabiting open and plains with sparse vegetation and rocky, soily substrates; prefers steppes. Its range extends from southern Balkan countries, Aegean and Mediterranean to SW Asia and Punjab in N. India; with a vertical distribution to 2000 m. (Baran & Atatur 1998).

To our knowledge, there are just two report of helminths in *Podarcis muralis;* Garcia-Adell and Roca (1988) reported 8 species of helminths from Spain including *Plagiorchis molini, Oochoristica* sp., *Mesocestoides* sp., *Skrjabinadon medinae, Spauligodon carbonelli, Skrjabinelazia pyrenaica, Skrjabinelazia* sp. and *Oswaldocruzia filiformis*. Kirin (2002a) reported 3 species of helminth from Bulgaria *Mesocestoides* spp., *Spauligodon extenuatus* and *Skrjabinelazia hoffmanni.*

There is just one report of helminth in *Podarcis siculus.* This study was conducted in Spain. Roca (1995) reported 6 species of helminths, *Paradistomum mutabile, Oochoristica gallica, Skrjabinadon medinae, Spauligodon cabrerae, Acuaria* sp. (larvae) and *Spirurida* gen sp.

In two reports related to *Ophisops elegans*, Goldberg and Bursey (2010) examined Iranian species and came across *Oochoristica tuberculata* and Nelli *et al*. (2014) encountered *Mesocestoides lineatus* in Armenian species. Nothing has been published on helminths of *P. muralis, P. siculus* and *O. elegans* from Bursa province, North-western Turkey. This study provides new helminth data for these lizard species from Bursa Province in Turkey.

* – corresponding author

Materials and Methods

A total of 80 lacertid lizards representing three species (*Podarcis muralis*, *Podarcis siculus* and *Ophisops elegans*) were examined for helminths. Thirty nine specimens of *P. muralis* (21 males, 18 females) were collected by hand in May 1997 – August 1998 from two different locations of Bursa Province, in Turkey. Number of lizards were n=17 at Sogukpınar, n=22 at Baraklı village. Sixteen specimens of *P. siculus* (6 males, 10 females) were collected by hand in May 1997 – July 1998 from two different locations of Bursa Province, in Turkey. Number of lizards were n=5 at İznik, n=11 at Fethiye village. Twenty one specimens of *O. elegans* (15 males, 4 females and 2 juvenils) were collected by hand in December 1996 – October 1998 from two different locations of Bursa Province, in Turkey. Number of lizards were n=6 at Karacabey, n=15 at Baraklı village. Lizards were humanely killed with sodium pentobarbital. The body cavity was opened and the digestive tract was removed. The esophagus, stomach, small and large intestines and lungs were opened and separately examined for helminths under a dissecting microscope. Helminths from each host were placed in individuals of ethanol for storage. For study, helminths were cleared in a drop of undiluted glycerol on a glass slide. Nematodes were identified from these temporary preparations. Digenea and Cestodes were fixed in 70 % ethanol, stained with iron-carmine, dehydrated, cleared and mounted in Entellan (Georgiev *et al*., 1986). Helminth identifications were based on the reference keys of Yamaguti (1961) and Schmidt (1986). Helminth voucher specimens were deposited in the helminth collection of Uludag University Museum of Zoology, Bursa, Turkey. Lizard specimens were deposited in the Department of Biology, Uludag University, Bursa, Turkey.

Ethical Approval and/or Informed Consent

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

Results

***Podarcis muralis* (Laurenti, 1768) Common Wall Lizard**
There were 5 species of helminths in these lizards.
***Plagiorchis elegans* (Rudolphi, 1802) Braun, 1902**
(Syn. *Fasciola elegans* Rudolphi, 1802; *Fasciola cirratus* Rudolphi, 1802; *Distoma colubri natricis* Rudolphi, 1809; *Distoma elegans* (Rudolphi, 1802) Rudolphi,1809; *Distoma colubri tessellati* Rudolphi, 1819; *Distoma lacertae* Rudolphi, 1819; *Distomum (Brachylaimus) elegans* (Rudolphi, 1802) Dujardin 1845; *Distomum erraticum* Linstow 1894; *Plagiorchis cirratus* (Rudolphi, 1802) Lühe, 1899; *Plagiorchis mentulatus* (Rudolphi 1819) Stossich, 1904; *Plagiorchis asperus* Stossich, 1904; *Plagiorchis notabilis* Nicoll, 1909; *Plagiorchis marii* Skrajabin, 1920; *Plagiorchis blumbergi* Massino 1927; *Plagiorchis brauni* Massino 1927; *Plagiorchis loossi* Massino 1927; *Plagiorchis massino* Petrov and Tichonoff , 1927; *Plagiorchis multiglandularis* Semenow, 1927; *Plagiorchis skrajabini* Massino 1927; *Plagiorchis uhlworni* Massino, 1927; *Plagiorchis potanini* Skrjabin, 1928; *Plagiorchis eutamiatis* Schulz, 1932; *Plagiorchis casarci* Mehra, 1937; *Plagiorchis ferrigunum* Mehra, 1937; *Plagiorchis eutamiatis* Zibethicus Vassiliev 1939; *Plagiorchis extremus* Strom, 1940; *Plagiorchis strictus* Strom, 1940; *Plagiorchis fuji* Ogata, 1941; *Plagiorchis ptschelkini* Sobolev, 1946; *Plagiorchis petrovi* Fediushin, 1949; *Plagiorchis oscineus* Sudarikov, 1950; *Plagiorchis castoris* Orloff et Moskalev, 1953; *Plagiorchis blatnensis* Chalupsky, 1954; *Plagirochis raabei* Furmaga, 1956; *Plagirochis stefanski* Furmaga, 1956, *Plagiorchis muris* sensu Prokopic and Genov, 1974; *Plagiorchis proximus* sensu Prokopic and Genov, 1974; *Plagiorchis cuculi* Schaldybin, Anikin, Budkin et Suslova, 1977)
Prevalence and mean intensity: 11/39 (28 %), 3 ± 2.38, 1 – 8.
Temporal distribution: 19 July 1998, 7 host with 2, 8, 1, 6, 4, 1, 1 respectively; 20 July 1998, 4 host with 2, 1, 3, 5 respectively.
Site of infection: Small intestine.
Type host and type locality: House sparrow, *Passer domesticus*, Germany (Rudolphi, 1802). ***Additional Turkish records:*** None.
Other reported hosts: **Amphibia:** yellowbelly toad, *Bombina variegata*, (Prokopic & Krivanec, 1975); pool frog, *Pelophylas lessonae*, (reported as Rana esculenta, Prokopic & Krivanec, 1975); common frog, *Rana temporaria*, (Capuse, 1971); **Reptilia:** sand lizard, *Lacerta agilis*, (Shevechenko & Barabashova, 1958; Moravec, 1963; Capuse, 1971; Lewin, 1992a; Shimalov *et al*., 2000; Sharpilo *et al*., 2001; Borkovcova & Kopriva, 2004); European green lizard, *Lacerta viridis*, (Capuse, 1971); viviparous lizard, *Zootoca vivipara*, (reported as *Lacerta vivipara*, Lewin, 1992b; Shimalov *et al*., 2000); European grass snake, *Natrix natrix* (Capuse, 1971); **Aves:** northern goshawk, *Accipiter gentilis*, (Sitko, 1998); Eurasian sparrowhawk, *Accipiter nisus*, (Sitko, 1998); spotted sandpiper, *Actitis macularius*, (Didyk *et al*., 2007); Balkal tean, *Anas formosa*, (Bykhovskaya-Pavlovskaya, 1962); mallard, *Anas platyrhynchos*, (Styczynska-Jurewicz, 1962); little stint, *Calidris minuta*, (Bykhovskaya-Pavlovskaya, 1962); twite, *Carduelis flavirostris*, (Massino, 1929); ruddy shelduck, *Casarca ferruginea*, (Mehra, 1937); black tern, Chlidonias nigra, (Massino, 1929); western marsh harrier, *Circus aeruginosus*, (Bykhovskaya Pavlovskaya, 1953; Krasnolobova, 1987); northern harrier, *Circus cyaneus*, (Krasnolobova, 1987); pallid harrier, *Circus macrourus*, (Bykhovskaya-Pavlovskaya, 1953; Krasnolobova, 1987); common quail, *Coturnix coturnix*, (Bykhovskaya-Pavlovskaya , 1953); common raven, *Corvus corax*, (Massino, 1927); carrion crow, *Corvus corone*, (Mühling, 1896); rook, *Corvus frugilegus*, (Braun, 1902); Eurasian jackdaw, *Corvus monedula*, (Massino, 1927); corncrake, *Crex crex*, (Macko, 1969); common cuckoo, *Cucullus canorus*, (Dubinia & Kulakova, 1960); common house-martin, *Delichon urbica*, (Odening, 1961); great spotted woodpecker, *Dendrocopos major*, (Styczynska-Jurewicz, 1962); merlin, *Falco columbarius*, (Massino, 1927; Krasnolobova, 1987); peregrine falcon, *Falco*

peregrinus, (Krasnolobova, 1987); Eurasian hobby, *Falco subbuteo,* (Bykhovskaya-Pavlovskaya, 1953; Styczynska-Jurewicz, 1962; Krasnolobova, 1987; Ferrer *et al*., 2004); Eurasian kestrel, *Falco tinnunculus*, (Sitko, 1998); red-footed falcon, *Falco vespertinus,* (Styczynska-Jurewicz, 1962; Krasnolobova, 1987); common chaffinch, *Fringilla coelebs*, (Bykhovskaya-Pavlovskaya , 1962); common snipe, *Gallinago gallinago*, (Massino, 1927); chicken, *Gallus gallus domesticus*, (Odening, 1959); Eurasian jay, *Garrulus glandarius*, (Bykhovskaya-Pavlovskaya, 1953); collared pratincole, *Glareola pratincola*, (Braun, 1902; Bykhovskaya-Pavlovskaya, 1962); barn swallow, *Hirundo rustica*, (Odening, 1961); red-backed shrike, *Lanius collurio*, (Massino, 1927); herring gull, *Larus argentatus*, (BykhovskayaPavlovskaya, 1962); great black-headed gull, *Larus ichthyaetus*, (Mhaisen *et al*., 1990); common blackheaded gull, *Larus ridibundus*, (BykhovskayaPavlovskaya, 1962); Hudsonian godwit, *Limosa haemastica* (Kinsella *et al*., 2007); black-tailed godwit, *Limosa limosa*, (Bykhovskaya-Pavlovskaya, 1962); Eurasian black grouse, *Lyrurus tetrix*, (BykhovskayaPavlovskaya, 1962); Eurasian swift , *Micropus apus*, (Odening, 1961); black kite *Milvus migrans*, (Krasnolobova, 1987); white wagtail, *Motacilla alba*, (Bykhovskaya-Pavlovskaya, 1962); yellow wagtail, *Motacilla flava*, (Bykhovskaya-Pavlovskaya, 1962); spotted flycatcher, *Muscicapa striata*, (Styczynska Jurewicz, 1962); Eurasian curlew, *Numenius arquata*, (Bykhovskaya-Pavlovskaya, 1962); slender-billed curlew, *Numenius tenuirostris*, (BykhovskayaPavlovskaya, 1962); tufted duck, *Nyroca fuligula*, (Styczynska-Jurewicz, 1962); Eurasian golden oriole, *Oriolus oriolus*, (Bykhovskaya-Pavlovskaya, 1962); Eurasia scops owl, *Otus scops*, (Braun, 1902); osprey, *Pandion haliaetus*, (Krasnolobova, 1987); bearded reeding, *Panurus biarmicus* (BykhovskayaPavlovskaya, 1962); great tit, *Parus major*, (Braun, 1902; Bykhovskaya-Pavlovskaya, 1962); house sparrow, *Passer domesticus*, (Braun, 1902); Eurasian sparrow, *Passer montanus*, (BykhovskayaPavlovskaya, 1962); coal tit, *Periparus ater*, (Massino, 1929); honey buzzard, *Pernis apivorus*, (Ferrer *et al*., 2004); ruff , *Philomachus pugnax*, (Bykhovskaya-Pavlovskaya, 1962); black-billed magpie, *Pica pica*, (Braun, 1902); glossy ibis, *Plegadis falcinellus*, (Bykhovskaya-Pavlovskaya, 1962); dunnock, *Prunella modularis*, (Styczynska-Jurewicz, 1962); Eurasian nuthatch, *Sitta europaea*, (StyczynskaJurewicz, 1962); common tern, *Sterna hirundo*, (Bykhovskaya-Pavlovskaya, 1962); common starling, *Sturnus vulgaris*, (Bykhovskaya-Pavlovskaya, 1953); barred warbler, *Sylvia nisoria*, (BykhovskayaPavlovskaya, 1962); hazel grouse, *Tetrastes bonasia*, (Bykhovskaya-Pavlovskaya, 1962); wood sandpiper, *Tringa glareola*, (Bykhovskaya-Pavlovskaya, 1962); fieldfare, *Turdus pilaris* (Bykhovskaya-Pavlovskaya, 1962); hoopoe, *Upupa epops*, (BykhovskayaPavlovskaya, 1962); **Mammalia:** arctic fox, *Alopex lagopus*, (Malczewski, 1961; Rausch *et al*., 1983); striped field mouse, *Apodemus agrarius*, (Furmaga, 1956; Zarnowski, 1960; Shimalov, 2002); yellownecked mouse, *Apodemus flavicollis*, (Matskasi, 1971); wood mouse, *Apodemus sylvaticus*, (Furmaga, 1956); dog, *Canis familiaris*, (Petrov & Tichonoff , 1927; Desrochers & Curtis, 1987); bank vole, *Clethrionomys glareolus*, (Matskasi, 1971; Tenora *et al*., 1983; Mazeika *et al*., 2003); cat, *Felis domesticus*, (Petrov & Tichonoff , 1927); harvest mouse, *Micromys minutus*, (Matskasi, 1971); common vole, *Microtus arvalis*, (Chalupsky, 1954); house mouse, *Mus musculus*, (Odening, 1959); water shrew, *Neomys fodiens*, (Panov & Karpenko, 2004); muskrat, *Ondatra ziabethicus*, (Sey, 1965; Matskasi, 1971); common shrew, *Sorex araneus*, (Matskasi, 1971).

Geographic range: Northern hemisphere.

Remarks: All species of *Plagiorchis* use aquatic snails as first intermediate hosts and insects as second intermediate hosts (Roberts & Janovy, 2000). Given the broad host-range any insectivore might be expected to harbor *Plagiorchis elegans. P. muralis* represents the second host record for *P. elegans*.

***Mesocestoides* sp. (tetrathyridium);**

Prevalence and range: 1 of 39 (3 %), 32.

***Temporal distribution*:** 2 June 1997, 1 host with 32.

***Site of infection*:** Body cavity.

Additional Turkish records: *Apathya cappadocica* (Birlik *et al*., 2015); *Anatololacerta danfordi* (Gürelli *et al*. 2007); *Darevskia rudis* (Birlik *et al*., 2018a); *Darevskia valentini* (Birlik *et al*., 2018b); *Lacerta trilineata* (Yıldırımhan *et al*., 2011); *Phoenicolacerta laevis* (Birlik *et al*. 2016).

Other reported reptilian hosts: The genus *Mesocestoides* is cosmopolitian and tetrathyridia can be found in all classes of vertebrates. We have listed known accidental or paratenic hosts reported from the Palearctic biogeographic region: Slow worm, *Anguis fragilis* (Lewin, 1990); Mongolian racerunner, *Eremias argus* (Dugarov *et al*., 2018); Sand lizard, *Lacerta agilis* (Nelli *et al*., 2014; Lewin, 1992a; Sharpilo *et al*., 2001); Redbelly rock agama, *Paralaudakia erythrogaster* (reported as *Agama erythrogaster*, Radchenko, 1973); Eastern giant emerald lizard, *Lacerta media* (Nelli *et al*., 2014); Iberian emerald lizard, *Lacerta schreiberi* (Roca & Ferragut, 1989); *Lacerta viridis* (Biserkov & Kostadinova, 1998); Snake-eyed lizard, *Ophisops elegans* (Nelli *et al*., 2014); Secret toadhead agama, *Phrynocephalus mystaceus* (Ikromov & Cho, 2004); Bocage's wall lizard, *Podarcis bocagei* (Roca *et al*., 1989); Iberian wall lizard, *Podarcis hispanica* (Roca *et al*., 1989); Common wall lizard, *Podarcis muralis* (Kirin, 2002a); Ibiza wall lizard, *Podarcis pityusensis* (Roca & Hornero, 1991; Roca & Hornero, 1994); Spanish psammodromus, *Psammodromus hispanicus* (Roca *et al*., 1986a; Roca & Lluch, 1988); Tenerife wall gecko, *Tarentola delalandii* (Roca *et al*., 1987); Smooth snake, *Coronella austriaca* (Biserkov, 1996); Western whip snake, *Hierophis viridiflvus* (Santoro *et al*., 2013); Aesculapean snake, *Zamenis longissimus,* (reported as *Elaphe longissima,* Biserkov, 1996); Halys pit viper, *Gloydius halys* (reported as *Ancystrodon halys,* Bogdanov *et al*., 1969); European grass snake, *Natrix natrix* (Lewin, 1992b); nose-horned viper, *Vipera ammodytes* (Biserkov, 1995).

Geographic range: Cosmopolitan (McAllister *et al*., 1991).

Remarks: The life cycle of *Mesocestoides* spp. is thought to require 3 hosts, i.e. a vertebrate definite host, a vertebrate second

intermediate host, and a purported arthropod first intermediate host (Rausch, 1994). Tetrathyrida are frequently found in the body cavities of amphibians, reptiles, birds and mammals (Padgett & Boyce, 2004). *P. muralis* represents the eighth host record for the genus *Mesocestoides* in Turkey.

***Skrjabinelazia hoffmanni* Li, 1934**

Prevalence, mean intensity and range: 4 of 39 (10 %) 3 ± 3.5, 1 – 8

Temporal distribution: 2 June 1997, 2 host with 8, 1 respectively; 19 July 1998, 1 host with 1; 20 July 1998, 1 host with 1.

Site of infection: Small intestine.

Type host and type locality: Mongolia racerunner, *Eremias argus*, China (Li, 1934).

Additional Turkish records: *Darevskia rudis* (Roca *et al*., 2015a; Birlik *et al*., 2018a); *Darevskia valentini* (Birlik *et al*., 2018b); *Lacerta trilineata* (Yıldırımhan *et al*., 2011).

Other reports: Comb-toed gecko, *Crossobamon eversmanni* (Andrusko & Markov, 1956; Sharpilo, 1976); Azarbaijan lizard, *Darevskia raddei* (Host reported as *Lacerta raddei*, Khomustenko & Ataev, 1979); *Darevskia saxicola* (Host reported as *Lacerta saxicola*, Sharpilo 1976); *Eremias argus* (Li, 1934; Dugarov *et al*., 2018); Kirghiz racerunner, *Eremias nikolskii* (Sharpilo, 1976); *Lacerta agilis* (Sharpilo, 1976; Sharpilo *et al*., 2001); *Lacerta viridis* (Biserkov & Kostadinova, 1998); *Podarcis bocagei* (Galdon *et al*., 2006; Roca *et al*., 1990); Carbonell's wall lizard, *Podarcis carbonelli* (Galdon *et al*., 2006); *Podarcis hispanica* (Roca *et al*., 1990); Lilford's wall lizard, *Podarcis lilfordi* (Hornero & Roca, 1992a: Roca & Hornero, 1994); *Podarcis muralis* (Kirin, 2002a; Roca *et al*., 1990); Canary wall gecko, *Tarentola angustimentalis* (Roca *et al*., 1999); *Teratoscincus scincus* (Sharpilo, 1976).

Geographic range: Azerbaijan (Khomustenko & Ataev, 1979); Bulgaria (Biserkov & Kostadinova, 1998); Central Asia (Andrusko & Markov, 1956); China (Li, 1934); Portugal (Galdon *et al*., 2006); Russia (Dugarov *et al*.,2018); Spain (Roca *et al*., 1990); Turkey (Yıldırımhan *et al*., 2011); Ukraine (Sharpilo *et al*., 2001).

Remarks: The life history of *S. hoffmanni* apparently has not been studied. However the cogener *S. galliardi* is claimed by Chabaud *et al*. (1988) to produce two types of egg, one thin-shelled and containing third-stage larva, probably autoinfective and a seond, red, thicker shelled with third-stage larvae which probably pass out of the host. *P. muralis* represents the fourth reptilian host record for *Skrjabinelazia hoffmanni* in Turkey.

***Skrjabinodon medinae* (García-Calvente, 1948) Specian and Ubelaker, 1974**

(Syn. *Pharyngodon medinae* García-Calvente 1948; *Parathelandros medinae* [García-Calvente, 1948] Read and Amrein, 1953).

Prevalence, mean intensity and range: 6 of 39 (15 %) 3.5 ± 2.88, 1 – 8

Temporal distribution: 19 July 1998, 5 host with 7, 1, 3, 8, 6 respectively.

Site of infection: Large intestine.

Type host and type locality: *Lacerta muralis*, Spain (García-Calvente, 1948)

Additional Turkish records: *Apathya cappadocica* (Birlik *et al*., 2015); *Darevskia rudis,* Birlik *et al*., 2018a); *Darevskia valentini* (Birlik *et al*., 2018b); *Iranolacerta brandtii* (Birlik *et al*., 2017); *Lacerta trilineata* (Yıldırımhan *et al*., 2011), *Phoenicolacerta laevis* (Birlik *et al*., 2016).

Other reports: *Lacerta schreiberi* (Roca & Ferragut, 1989); *Podarcis bocagei* (Roca *et al*., 1989); *Podarcis hispanica* (Roca *et al*., 1986b; Roca & Lluch, 1988; Roca *et al*., 1989; Hornero & Roca, 1992a); Lilford's wall lizard, *Podarcis lilfordi* (Hornero & Roca, 1992b; Roca & Hornero, 1994); *Podarcis muralis* (Dollfus *et al*., 1961; Garcia-Calvente, 1948; Hornero & Roca, 1992a); *Podarcis pityusensis* (Roca & Hornero, 1991; Hornero & Roca, 1992a; Roca & Hornero, 1994); *Zootoca vivipara* (Host reported as *Lacerta vivipara*, Dollfus *et al*., 1961).

Geographic range: France (Dollfus *et al*., 1961); Spain (Roca & Hornero, 1994); Turkey (Yıldırımhan *et al*., 2011).

Remarks: *P. muralis* represents the seventh host record for the species *Skrjabinodon medinae* in Turkey.

***Spauligodon saxicolae* Sharpilo, 1961**

Prevalence, mean intensity and range: 2 of 39 (5 %) 18 ± 24, 1 – 35

Temporal distribution: 19 July 1998, 2 host with 1, 35 respectively.

Site of infection: Large intestine.

Type host and type locality: Scaly lizard, *Lacerta saxicola* (Sharpilo, 1962) Ukraine.

Additional Turkish records: *Darevskia bendimahiensis* (Roca *et al*., 2015a); *Darevskia clarkorum* (Roca *et al*., 2016)*; Darevskia parvula* (Roca *et al*., 2016); *Darevskia radde* (Roca *et al*., 2016)*; Darevskia rudis* (Roca *et al*., 2016; Murvanidze *et al*., 2008); *Darevskia sapphirina* (Roca *et al*., 2015b); *Darevskia unisexualis* (Roca *et al*., 2016); *Darevskia uzzelli* (Roca *et al*., 2015b); *Darevskia valentini* (Roca *et al*., 2016); *Eremias strauchi* (Düşen *et al*., 2013); *Eremias suphani* (Düşen *et al*., 2013); *Mesalina brevirostris* (Düşen *et al*., 2016).

Other reports: *Eremias velox* (Ikromov & Cho, 2004); *Darevskia caucasica* (Uhlírová, 2005); *Lacerta strigata* (Murvanidze *et al*., 2008); *Daraevskia saxicola* (Goldin, 1975; Murvanidze *et al*., 2008); *Darevskia rudis* (Murvanidze *et al*., 2008); *Podarcis vaucheri* (Carretero *et al*. (2011); *Coluber jugularis* (Murvanidze *et al*., 2008).

Geographic range: Algeria (Carretero *et al*.,2011); Azerbaijan (Uhlirova, 2005); Crimea (Goldin, 1975); Georgia (Murvanidze *et al*., 2008); Turkey (Dusen *et al*. 2013).

Remarks: *P. muralis* represents the 12th reptilian host record for *Spauligodon saxicolae* in Turkey.

***Podarcis siculus* (Rafinesque-Schmaltz, 1810) Istanbul Wall Lizard**

One helminth species was found in the host.

***Spauligodon saxicolae* Sharpilo, 1961**

Prevalence, mean intensity and range: 12 of 16 (75 %), 33 ± 29.5, 4 – 115.

Temporal distribution: 5 May 1997, 2 host with 20, 30 respectively; 25 May 1998, 7 host with 30, 25, 32, 40, 6, 7, 43, 15 respectively; 24 June 1998, 2 host with 4, 60 respectively.

Site of infection: Large intestine.

Remarks: See remarks above under *Podarcis muralis*.

***Ophisops elegans* Menetries, 1832 Snake – eyed Lizard**

No helminths were found in the host.

Discussion

Sixteen (41 %) of 39 *Podarcis muralis* harbored 143 helminths representing 5 species: 10 lizards harbored 1 species, 5 harbored 2 species and 1 harbored 4 species. There were 8.7 ± 9.1 SD (range 1 – 32) helminth individuals per host lizard and 3.5 ± 0.5 SD helminth species per host lizard.

Twelve (67 %) of 16 *P. siculus* harbored 397 helminths representing 1 species: 12 lizards harbored 1 species. There were 33 ± 25.5 SD (range 6 – 115) helminth individuals per host and 24.8 ± 0.5 SD helminth species per host.

There are not helminths in the lizard of the *Ophisops elegans* species.

Of the 147 Turkish reptile species (Uetz, 2019) helminth lists are available for 24 species: *Acanthocadtylus harranensis* Baran, Kumlutas, Llanza, Sindaco, Avci and Crucitti, 2005; *Acanthodactylus schreiberi* Boulenger, 1878; *Anatololacerta danfordi* (Gunther, 1876); *Apathya cappadocica* (Werner, 1902); *Darevskia armeniaca* (Mehely, 1909); *Darevskia bendimahiensis* (Schmidtler, Eiselt and Darevsky, 1994); *Darevskia clarkorum* (Darevsky and Vedmederja, 1977); *Darevskia parvula* (Lantz and Cyren, 1913); *Darevskia raddei* (Boettger, 1892); *Darevskia rudis* (Bedriaga, 1886); *Darevskia sapphirina* (Schmidtler, Eiselt and Darevsky, 1994); *Darevskia unisexualis* (Darevsky, 1966); *Darevskia uzzellis* (Darevsky and Danielyan, 1977); *Darevskia valentini* (Boettger, 1892); *Eremias pleskei* Nikolsky, 1905; *Eremias strauchi* Kesslser, 1878; *Eremias suphani* Basoglu and Hellmich, 1986; *Iranolacerta brandtii* (De Filippi, 1863); *Lacerta trilineata* Bedriaga, 1886; *Lacerta viridis* (Laurenti, 1768); *Mesalina brevirostris* Blanford, 1874; *Parvilacerta parva* (Boulenger, 1887); *Phoenicolacerta laevis* (Gray, 18838); *Podarcis tauricus* (Pallas, 1814).

This report is the first report the helminth fauna list for *P. muralis* and *P. siculus* in Turkey (Table 1). However, additional studies will be required before the component community of helminths infecting Turkish lizards can be determined. For the 24 species listed above, there are on average 3.4 ± 3.3 SD (range 1 – 11) helminth species per lizard species. Currently, we can say that Turkish lizards are infected by generalist Nematodes, i.e. Nematode species that infect more than one host species. And also Turkish lizard is infected by some Digenea and Cestoda species.

Authors state no conflict of interest.

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Table 1. Helminths in lizards from Bursa-Turkey.

	P. muralis	*P. siculus*	*O. elegans*
DIGENEA			
Plagiorchis elegans	+	-	-
CESTODA			
Mesocestoides sp. (tetrathyridium)	+	-	-
NEMATODA			
Skrjabinodon medinae	+	-	-
Spauligodon saxicolae	+	+	-
Skrjabinelazia hoffmanni	+	-	-

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Case Report

Rare case of *Angiostrongylus vasorum* intraocular infestation in an asymptomatic dog

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Summary

The presented clinical observation shows an atypical case of *Angiostrongylus vasorum* intraocular infection in an 18-month-old male beagle from north-eastern Slovakia. The dog presented with a motile worm in the anterior chamber of the right eye. No ocular signs or symptoms of a systemic disease were observed. The faecal examination using Baermann´s technique and flotation was negative. Diagnosis was established following surgical removal of the worm. The specimen was determined as an *A. vasorum* female based on morphological features and confirmed by means of PCR technique and sequencing. To the best of our knowledge, the presented manifestation is the first ocular case of angiostrongylosis with absence of typical symptoms or signs of the disease.
Keywords: *Angiostrongylus vasorum*, French Heartworm, canine lungworm, ocular angiostrongylosis

Introduction

Angiostrongylus vasorum (Nematoda, Metastrongyloidea), commonly known as French heartworm, is a life-threatening nematode for dogs. General findings suggest that *A. vasorum* is occurred in isolated endemic areas. Since the first finding in Southern France in 1853 (Serres, 1854; Guilhon, 1963), the parasite was observed in Ireland (Roche & Kelliher, 1968), Switzerland (Wolff, 1969), Uganda (Bwangamoi, 1972), England (Simpson & Neal, 1982), Italy (Poli *et al*., 1984) and Denmark (Bolt *et al*, 1992). However, in recent years, autochthonous cases of infection are increasingly diagnosed in other European countries where the parasite previously did not occur, including Slovakia (Elsheikha *et al*., 2014).
The indirect life cycle of *A. vasorum* involves slugs and snails as intermediate hosts and frogs as paratenic hosts that harbour infective third stage larvae (L3). The definitive host becomes infected after ingestion of infected intermediate or paratenic host (Guilhon, 1963; Bolt *et al*., 1993).

The disease is most frequently presented as cardiorespiratory distress with a history of gagging, coughing, exercise intolerance and dyspnoea. Bleeding abnormalities, coagulopathy, neurological symptoms, general malaise, uveitis, depression, and anorexia are also described. Occasionally, cases of aberrant migration of *A. vasorum* to liver, pancreas, kidney even to CNS have been observed (Koch & Willesen 2009). The present study describes an atypical case of *A. vasorum* localisation in the anterior chamber of the right eye of an 18-month-old beagle from Slovakia.

Case Presentation

An 18-month-old beagle male from Bardejov, north-eastern Slovakia was referred to a private veterinary clinic in Prešov (Slovakia) for sudden appearance of a filiform foreign body in the right eye. The dog was kept indoors in the city, was usually walked in the vicinity of a river and never moved outside the region. Initial clinical ophthalmological examination revealed the presence of a

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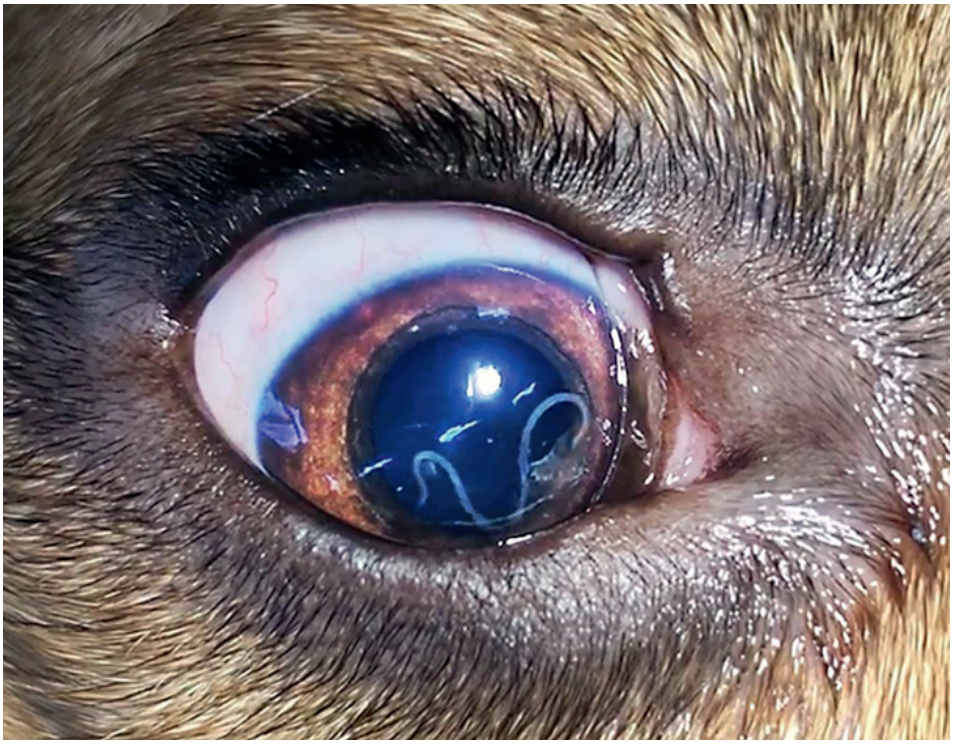

Fig. 1. Adult *Angiostrongylus vasorum* worm in the anterior chamber of the right eye of an 18-month-old male beagle from Slovakia.

motile intraocular nematode in the anterior chamber of the right eye (Fig. 1). Apart from the infection, the eye appeared ophthalmologically normal. Clinical examination revealed no evidence of a systemic disease. The faeces were investigated by the Baermann technique and flotation method with zinc sulphate solution (s.g. 1.2) (Bowman, 2014) with negative results. Other examinations were declined by the owner.

Surgical removal of the parasite was performed under injection anaesthesia (Dexmedetomidine, Diazepam, and Ketamine). Removal of the parasite was performed by anterior chamber paracentesis and aspiration of the worm using 0.9 mm port. A 2 % methyl cellulose solution was injected into the anterior chamber and the inputs were sutured with 7/0 absorbable filament, then the eye was *pro tem* surgically covered with third eyelid.

Postoperative medication consisted of topical treatment with steroid solution of fluoremetolon twice per day (Efflumidex Liquifilm Int Opu, Allergan Pharmaceuticals, Ireland), antibiotic instillation of tobramycinum four times per day (Tobrex Int Opo, S.A. Alcon-Couvreur, Belgium), and 1 % solution of atropine sulphate twice per day. The patient wore an Elizabethan collar until the stitches were removed day 4 post surgery. A follow up at day 4 revealed complete resolution without observable ophthalmologic after-effects seen in the right eye (Fig. 2). Specific anthelmintic treatment of angiostrongylosis consisted of single topical application of imidacloprid 250 mg/ moxidectin 62.5 mg (Advocate®,Bayer, Germany). A telephonic follow-up was performed six months post-surgery and the owner reported no evidence of systemic or ocular clinical signs.

Parasitological Findings

The extracted nematode was identified based on morphometric and characteristic morphological features according to species description by Costa *et al*. (2003) under the Leica DM4000B light microscope, Leica DFC 290 HD camera and Leica Application Suite V 3.8.0 software (Leica Microsystems GmbH, Germany).

The worm was identified as an adult female of *A. vasorum*. The body measured 24.3 mm in length and maximum body width was 0.534 mm. The buccal capsule directly joined the rhabditoid oesophagus with length 257.81 µm. The nerve ring was situated approximately in the middle of oesophagus. The excretory pore was located near oesophago-intestinal junction, 478.89 µm from the anterior extremity. The caudal end was ventrally curved and rounded. The vulva was situated 306.57 µm from the anus. The female cuticle was transparent. The ovarians filled with oocytes was twisted along the reddish intestine and created a “barber pole” appearance (Fig. 2).

The nematode was homogenized with 5 mm stainless beads (Qiagen®, Hilden, Germany) and ATL buffer in Qiagen TissueLyser (Qiagen®, Germany) for 30 Hz/6 min. DNA extraction was provided by a commercial isolation kit DNeasy Blood & Tissue (Qiagen®, Hilden, Germany), following the steps in protocol. A fragment of ITS2 rDNA was amplified by conventional PCR assay using the *A. vasorum*-specific primer set AV4/AV5 designed by Al-Sabi *et al*. (2010). A DNA fragment of 250 bp was separated on a 1.5 % agarose gel. The positive template was purified using NucleoSpin®

Fig. 2. Female *Angiostrongylus vasorum* extracted from the eye of a beagle dog from Slovakia. A - anterior extremity, B - caudal end (v-vulva, a - anus), C - Ovaria twisted along reddish intestine - a "barber pole" appearance, D - uterus

Gel and a PCR Clean-up kit (Macherey-Nagel GmbH & Co., KG, Germany) and sequenced by Sanger sequencing in both directions. Sequences were compared by BLAST (Basic Local Alignment Search Tool) with sequences available in GenBank.

A 156 bp long overlapping fragment of *A. vasorum* revealed 99 % similarity with the isolate obtained from a dog from Italy (KF270683). The nucleotide sequence of *A. vasorum* ITS2 rDNA gene fragment obtained during the study was deposited in GenBank under accession number MH018578.

Discussion and Conclusion

The herein presented description of *A. vasorum* specimen found intraocularly in a dog from north-eastern Slovakia is an atypical but not sole case of ectopic location of *A. vasorum* in the eye. The ocular localisation of the parasite has to date been reported several times as summarized by Colella *et al*. (2016), however this patient is to the best of our knowledge the first reported case where any ocular pathology and any other clinical signs associated with *A. vasorum* infection were absent. Dogs of all ages can be infected with *A. vasorum*, however, several studies showed that dogs younger that one year are more susceptible to clinical infection, presumably due to riskier behaviour when scavenging or playing with snails (Chapman *et al*. 2004; Koch & Willesen, 2009). This is true also for the herein reported case where the dog was in the age of 18 months and the owner confirmed its curiosity regarding snails and frogs.

The previously reported 8 cases of ocular *A. vasorum* migration originated from endemic countries such as France, Great Britain, Denmark, Canada and Italy (Colella *et al*., 2016). In Slovakia, the first canine angiostrongylosis cases were reported in 2012 and 2013 from eastern Slovakia (Hurníková *et al*. 2013; Miterpáková *et al*. 2014). The following copro-epidemiological research in the territory of Slovakia revealed a relatively high prevalence of *A. vasorum* in dogs and red foxes (4.13 % and 5.43 %, respectively) (Miterpáková *et al*. 2015; Čabanová *at al*. 2018a, 2018b).

Despite the fact that angiostrongylosis is usually presented with cardio-respiratory clinical signs, the majority of infected dogs identified within the above mentioned study were asymptomatic. Also here presented ocular localisation of *A. vasorum* reveals that angiostrongylosis should be included into differential diagnosis of unexplained canine eye disorders.

Ethical Approval and Informed Consent

No animals were killed for the purpose of this study.

Conflict of interest

The authors declare that they have no conflict of interest**.**

Acknowledgements

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Case Report

Concurrent helminthosis engendered gastroenteritis in a leopard *Panthera pardus*

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Article info

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Summary

The necropsy of a leopard (*Panthera pardus*), succumbed to a chronic ailment exhibited a mixed parasitic gastroenteritis. Gross internal examination of carcass revealed the presence of round and tapeworms in the stomach and intestines with diffuse catarrhal and hemorrhagic gastroenteritis. The detailed examination of the intestinal content revealed the presence of *Toxocara canis* and *Spirometra* species eggs. Also, the gross morphological investigation of round and tapeworms approved the presence of both species. Histo-pathological examination showed sloughing of intestinal epithelium, hemorrhages, and ulcerative areas with the infiltration of polymorphonuclear cells admixed with mononuclear cells. Lungs revealed the accumulation of eosinophilic edematous fluid in the alveolar spaces along with inflammatory cells. These parasites are pathogenic to precious wild felids and often pose a threat of zoonotic transmission due to spill-over infections. The present case study is an attempt to put on record a case of parasitic gastroenteritis in a captive leopard.
Keywords: Leopard; parasitic diseases; *Spirometra* species; *Toxocara canis*; zoonosis

Introduction

Out of 250 wild carnivore species distributed throughout the world, 60 species are recorded from India (Acharjyo, 2004). The main purpose of keeping the wild carnivores in captive state in zoological/ wildlife parks is associated with education, exhibition and gene conservation (Khatun *et al*., 2014). In natural habitat, wild animals sustain in a balanced system with the parasites due to some natural resistance (Thawait *et al*., 2014). Whereas, captivity leads to stress further ensuing depressed immune state of the wild animals, eventually rendering them vulnerable to various infectious diseases including parasitic, bacterial and viral (Moudgil *et al*., 2013). Helminth parasites, if present in heavy numbers are often capable to cause mortality and morbidity in wild captive animals (Acharjyo, 2004). Also, certain helminths infecting the wild animals especially carnivores hold significant zoonotic potential. Wild animals are quite potent to spill over the infection to other animals, humans and birds as well (Otranto *et al*., 2015). The transmission of parasites from wild animals to domestic animals and human beings is mostly a result of constricting the boundaries meant for the wild animals. So, domestic animals and human population could easily pick the infection at the close vicinity of national parks, wildlife sanctuaries and zoological/ wildlife parks etc. and thereby wild animals can act as a potent mode of disease transmission (Singh *et al*., 2017).

Geo-helminths could be considered as most potent parasitic invaders of wild animals in captivity rather than bio-helminths as they get optimum conditions for development and can quickly lead to re-infection (Panayotova-Pencheva, 2013). Toxocariasis in wild felids is an important parasitic disease which can affect any age group (Despommier, 2003) and leads to neurotoxocarosis in human beings which often act as precipitation factor for the de-

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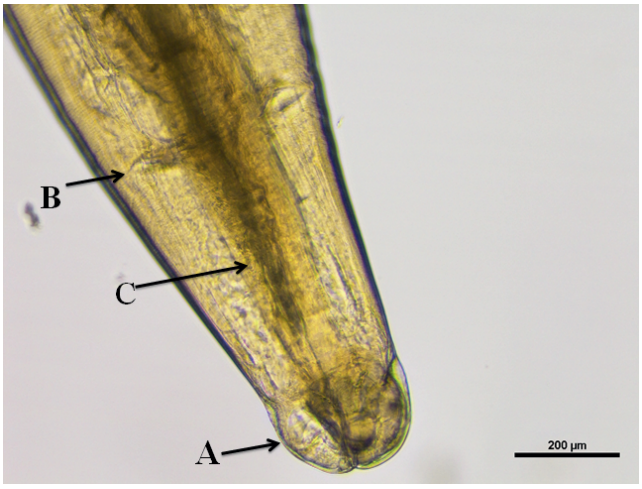

Fig.1. Photomicrograph of anterior end of *Toxocara canis* showing triradiate lips (A), cervical alae (B) and oesophagus (C) (10×)

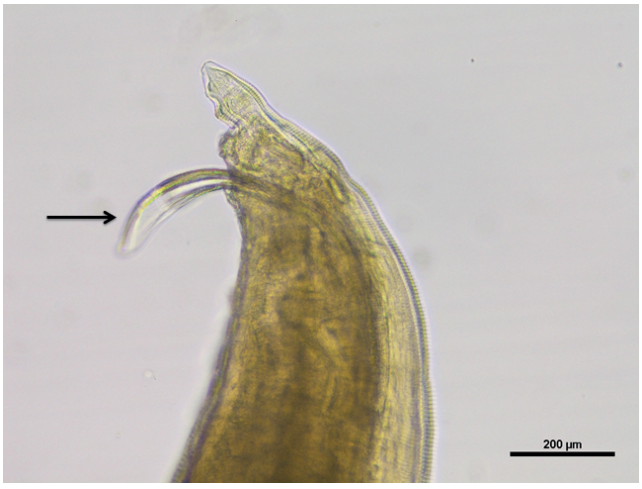

Fig. 2. Photomicrograph of posterior end of male *T. canis* showing sub equal spicules (arrow) (10×)

velopment of epilepsy (Xinou *et al*., 2003). *Toxocara* species has earlier been reported sporadically in leopards of various zoological parks of India during coprological and necropsy investigations (Nashirudullah & Chakraborty, 2001; Singh *et al*., 2006; Mahali *et al*., 2010; Thawait *et al*., 2014). Similarly, *Spirometra* species was also recovered during a necropsy of a leopard in a forest in Shimoga, Karnataka (Ananda *et al*., 2011). The highest incidence of spirometrosis in wild felids is associated with consumption of intermediate hosts including tadpoles, snakes, birds and alligators (Arjun *et al*., 2017). The parasitic load in hosts can lead to low fertility, decline in body weight, heavy morbidity and mortality. Thus, the present study is an attempt to highlight the presence and pathological aftermaths of concurrent helminthosis in precious wild felid *Panthera pardus* in India.

Material and Methods

A captive male leopard, approximately 19 years old weighing 35 kg was maintained at Gopalpur Zoological Park, Himachal Pradesh. A thorough clinical examination of the leopard showed that the animal was debilitated, anorectic, anemic and showed respiratory distress. The blood sample was collected for hemato-biochemical examination from tail vein of the animal after tranquilization with injection Xylazine and Ketamine @ 1mg/kg body weight and 5 mg/kg body weight respectively through intramuscular route.

The hematological parameters considered for analysis included hemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), total leukocyte count (TLC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). The serum biochem-

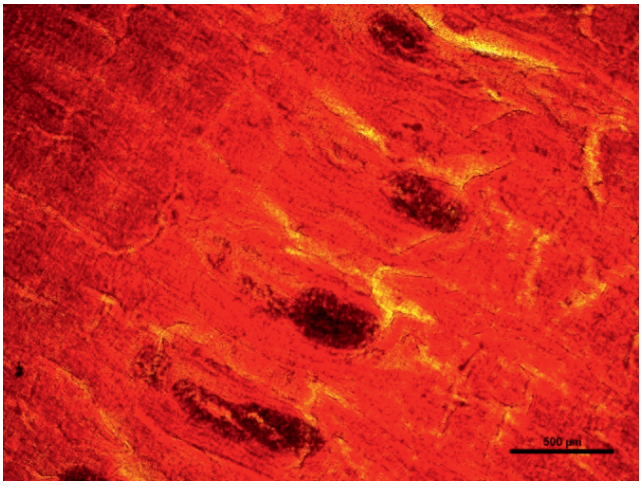

Fig. 3. Photomicrograph of gravid proglottid of *Spirometra* species exhibiting spiralled uterus (4×)

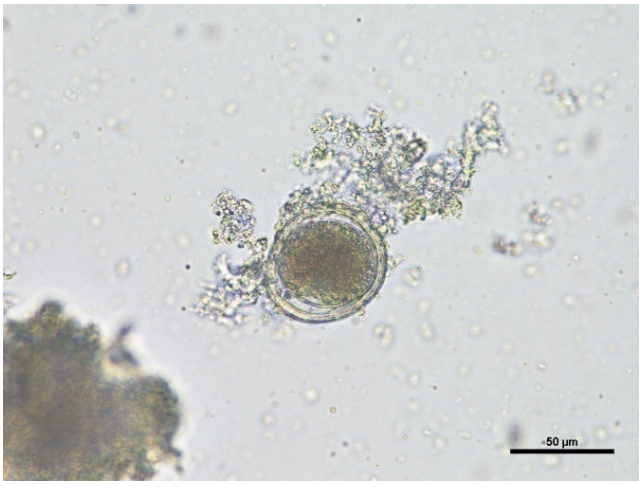

Fig. 4. Photomicrograph of unembryonated *Toxocara canis* egg (40×)

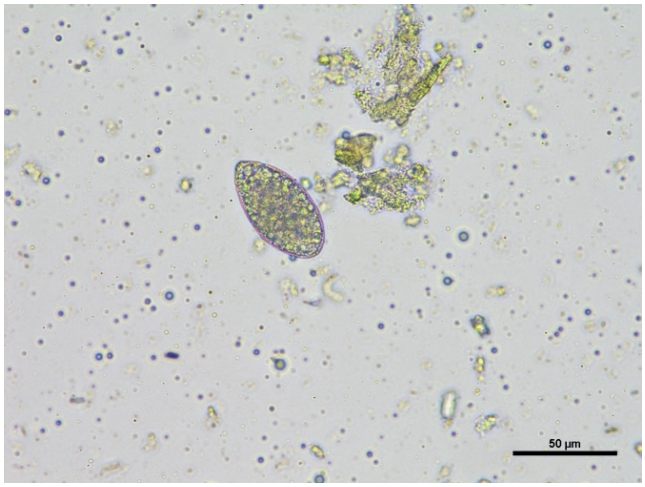

Fig. 5. Photomicrograph of *Spirometra* species egg with pointed ends (40×)

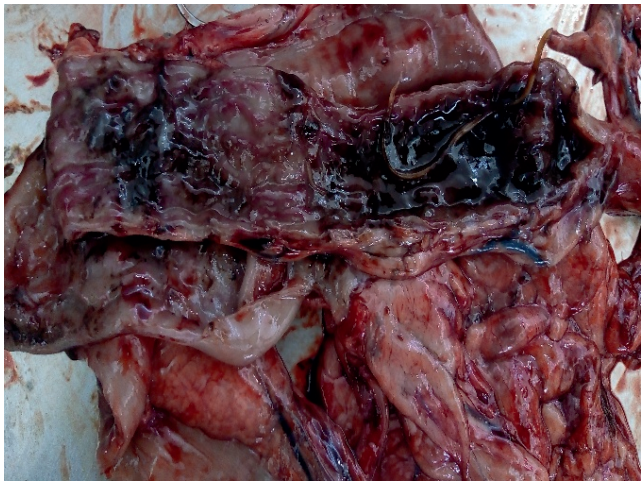

Fig. 6. Intestinal mucosa showing presence of round worms along with catarrhal to haemorrhagic exudate

ical parameters included alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, blood urea nitrogen (BUN) and creatinine. The animal was treated symptomatically but not responded to the treatment and succumbed to the infection.

The leopard was presented to the Department of Veterinary Pathology, DGCN COVAS CSKHPKV, Palampur for necropsy examination. A thorough external and internal examination of the animal was performed for the presence of any injury, ectoparasites and other associated pathological alteration. The gross lesions were recorded after detailed necropsy examination. On examination of gastrointestinal system the presence of round and tapeworms were evident. The parasites were removed gently, washed in normal saline and sent to the Department of Veterinary Parasitology for species identification. The nematodes were cleared in lactophenol in order to assess the morphological and morphometric characteristics of males and females (Zajac, 1994). The length and width of roundworms was measured in millimetres (mm) and was expressed considering mean ± standard deviation. The cestodes were stained with Borax carmine stain as per the method of Urquhart *et al*. (1996). The smears prepared from the intestinal contents revealed the presence of eggs. The morphometric analysis targeting the size of the eggs was performed as per Kazacos & Turek (1983). The length and breadth of the eggs was expressed as mean ± standard deviation in micrometers. The organs showing pathological changes were collected and fixed in 10% neutral buffered formalin for histopathological examination. The fixed tissues were embedded in paraffin, sectioned at 4 – 5 microns and stained with Haematoxylin and Eosin as per the protocol given by Luna & Lee (1968).

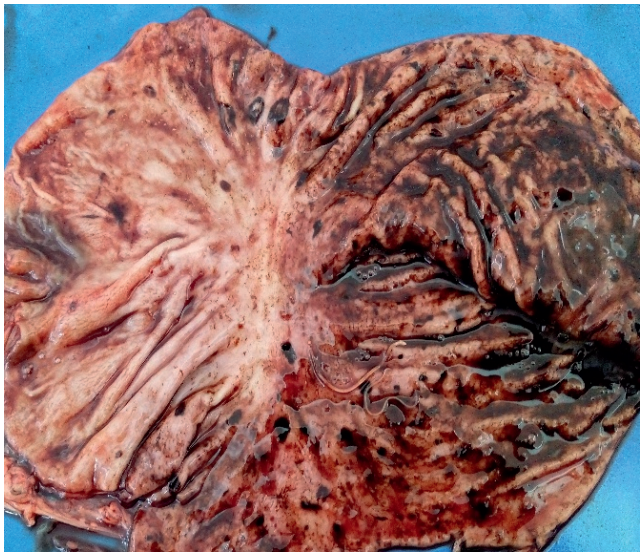

Fig. 7. Thickened gastric mucosa along with multifocal erosive and ulcerative areas

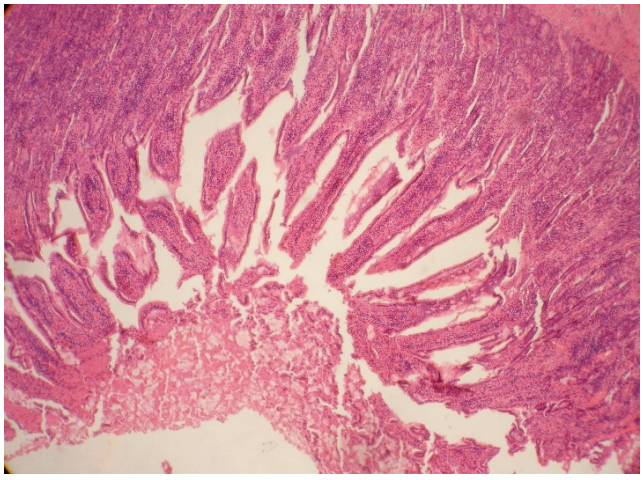

Fig. 8. Photomicrograph showed denuded intestinal epithelium, homogenous pink catarrhal exudates and mononuclear cells admixed with few neutrophils (40×)

Ethical Approval and/or Informed Consent

No experimental animals were used in this study.

Results and Discussion

In the past, only sporadic cases of parasitic infections during scatological and necropsy investigations have been reported in wild felids from different parts of India and there is a woeful paucity of comprehensive studies involving pathological upshots. Parasitic diseases reported in wild animals mainly include infections due to gastrointestinal parasites (Singh *et al*., 2006) and haemoprotozoans.

In the present case study, the body temperature of the leopard was observed 96.6° F. The respiration rate and heart rate were 16/min and 92 beats/ min, respectively. The Hb and PCV concentration observed in leopard's blood was 7.7 g/dl and 25% respectively, which were lower than the normal reference values as given by Sabapara *et al.* (2008). WBCs count reported was 13.3×10^9/L (higher than normal), whereas the total erythrocyte count (TEC) was 5.17×10^{12}/L (normal) compared with the reference values given by Sabapara *et al*. (2008). The platelet count obtained was 513×10^9/L, which was higher than the reference values given by Salakij *et al*. (2009). The results of erythrocytic indices were MCV-48.4 fl, MCH-14.8 pg, and MCHC-30.8 g/dl. On biochemical analysis of serum sample, no statistically significant alterations in the values of glucose (101 mg %), protein (6.2 g/dl) and creatinine (1.9 mg) were observed, whereas the values of blood urea nitrogen (100.4 mg %) was elevated from the normal reference values. The values of liver specific enzymes ALT (51 IU/L), ALP (18 IU/L), AST (30 IU/L) and GGT (5 IU/L) were almost normal as compared with the reference values given by Singh (2005). The variations in the values of hemato-biochemical parameters, reduced bone density on radiographic examination, enlarged kidneys and shrunken liver lobes might be an outcome related to the effect of age.

The results obtained from parasitological investigation showed that the leopard was infested with intestinal *Toxocara canis* and *Spirometra* spp. The adult worms of *Toxocara canis* were identified on the basis of gross morphological examination. Grossly, the size of the male worms (n=6) were measured 67.14 ± 4.26 mm (61.6 – 71.8 mm) × 1.28 ± 0.08 mm (1.2 – 1.4 mm) (length × width), whereas, female parasites (n=6) were measured 86.88 ± 3.92 mm (81.2 – 90.8 mm) × 1.72 ± 0.11 mm (1.6 – 1.8mm) (length × width), respectively. The distinctive morphological features included three developed triradiate lips (one dorsal and two subventral) with cervical alae and filariform oesophagus (Fig. 1) in adult parasites of *T. canis*. The caudal end of the male parasites possessed two subequal spicules with large one 2.16 ± 0.29 mm (1.8 – 2.4 mm) and smaller one 1.12 ± 0.08 mm (1 – 1.2 mm) (Fig. 2), respectively; whereas, female posterior end had a tapering blunt tail. The findings were in concordance with the observations of Radwan *et al*. (2009), who ascertained the prevalence of *Toxocara* species in wild animal population based on morphological studies. *Toxocara* species parasites had also been earlier reported from wild felids (including leopards) from different zoological gardens of India (Moudgil *et al*. 2015) and heavy burdens of these parasites had also been incriminated for mortalities of the infested captive wild animals. In case of cestode parasites, mature proglottids were broader than long and in gravid proglottids, numerous ovoid eggs with pointed ends were observed in the spiralled uterus (Fig. 3). The cestodes were identified as a species of *Spirometra* described by Yamaguti (1959). *Spirometra* species had also been earlier reported from wild felids kept in captivity from different parts of India (Moudgil *et al*. 2015). The intestinal content smears revealed the presence of two types of eggs; first, subglobular ascarid eggs with thick, finely pitted shell and round embryonic mass and second, unembryonated ovoid yellowish-brown eggs with pointed ends. The ascarid egg size (n=10) was 89.9 ± 3.07 µm (86.2 – 94.8 µm) × 75.3 ± 1.76 µm (72.8 – 78.2 µm) (length × breadth) (Fig. 4); whereas ovoid cestode eggs measured 59.9 ± 2.19 (56.8 – 62.4 µm) × 35.1 ± 0.95 µm (33.6 – 36.2 µm) (Fig. 5). The morphometric observations of the eggs of ascarids and cestodes substantiating to be of *Toxocara canis* and *Spirometra* species were in concordance with morphometric values reported by Brooker & Bundy (2014); Soulsby (1982); Muller-Graf (1995); Zajac & Convoy (2012), respectively.

The infection of wild felids with ascarids could be attributed to the housing conditions, especially the floors. In case of soil or wooden floors, the fecal material of the animals either remains clogged or attached to the surface (Moudgil *et al.*, 2017). The conditions lead to survivability of the eggs for a longer time even in harsh environmental conditions, eventually leading to transmission of infection to susceptible animals (Bowman, 1999; Singh *et al*., 2006). The presence of direct life cycle of the ascarids and short generation period for the infective stages could be considered as a reason for persistence of ascarid infection in well sanitized cages (Bowman, 1999; Moudgil *et al*., 2014). On the other hand, in case of *Spirometra* species a wide variety of animals and birds act as second intermediate hosts containing the plerocercoid stages (Soulsby, 1982) and consumption of any such intermediate host could have resulted in infection to the leopard.

The detailed necropsy examination of the leopard showed edematous and diffusely congested lungs with scanty frothy exudates in trachea. A heavy load of adult creamish white round worms was present in the stomach. The mucosa of the stomach was thickened and showed multifocal areas of erosions and ulcerations (Fig. 7). On opening the intestine, off dull white colored round worms and tapeworms were seen. The mucosa of intestinal loops containing these worms showed catarrhal to hemorrhagic enteritis (Fig. 6).

Histopathologically, stomach revealed denudation of mucosa, indicating ulcerative lesions, areas of diffuse hemorrhages along with the infiltration of mononuclear cells (MNCs). The small intestine exhibited denudation and clubbing of villi, homogenous pink catarrhal exudates with area of hemorrhages, cellular debris and

inflammatory cells especially MNCs admixed with few neutrophils (Fig 8). Eosinophilic edematous fluid was accumulated in the alveolar spaces of the lungs along with the infiltration of PMNCs admixed with MNCs. The histological picture of spleen revealed the presence of depleted lymphoid follicles, which is a strong indication of immunosuppression.

The hematological and biochemical parameters are reliable indicators of the health status of the animals (Ohaeri & Eluwa, 2011) and may prove important in subclinical and clinical infections. The decline in hematological parameters like Hb and PCV, which is important for causing anemia and hypoproteinaemia in the leopard is unclear. However, some of the researchers believe that oxidative stress and lipid peroxidation mechanisms of tissue damage could be the most appropriate cause of anemia in ascarid infections (Salem *et al*., 2015).

Most of the wild animals are endangered and already at the verge of extinction due to habitat destruction (forest fire), loss of genetic diversity, improper feeding and hunting (Sengar *et al*., 2017). The pace of this mechanism is further exacerbated by many diseases caused by a variety of pathogenic agents including parasites. The health status of captive wild felines is often influenced by various factors including age, feeding, environment, sanitation and irregular deworming which increases the risk of parasitism. The parasites (nematodes and cestodes) observed in the present study apart from inflicting serious health hazards and even mortalities of the animals also hold significant zoonotic potential. The present necropsy study suggests the necessity of regular deworming in captive wild animals and emphasizes on rising trends of parasitic infestations which are often overlooked.

Conflict of Interest

The authors declare that they have no conflict of interest regarding the publication.

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Research Note

Stray dogs of Sofia (Bulgaria) could be an important reservoir of heartworm (*Dirofilaria immitis*)

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Summary

Dirofilaria immitis (heartworm) is a zoonotic and an emerging disease, expanding in Europe. In Bulgaria, the presence of the parasite has been described in many regions. However, canine heartworm has hardly been evaluated in the capital of the country and, therefore, the aim of this study was to evaluate the prevalence and distribution of canine heartworm in Sofia. Eighty stray dogs from the city of Sofia and the metropolitan area were analysed for circulating *D. immitis* antigens. The prevalence was 31.25 %, being 34.7 % in the metropolitan area and 25.8 % in the city of Sofia. The current results are among the highest reported in the country. This could be due to the lack of prophylactic measures against infection in these dogs, but also to the spread of *D. immitis* into non-endemic countries. Stray dogs may act as an important reservoir of heartworm being a risk for client-owned animals and for the development of pulmonary dirofilariosis in inhabitants. The results show the need to establish further epidemiological studies and prophylactic campaigns for stray and client-owned animals, as well as to create awareness campaigns about the severity and importance of this disease for both animals and humans.

Keywords: *Dirofilaria immitis*; zoonosis; heartworm; dirofilariosis; epidemiology; seroprevalence

Introduction

Dirofilaria immitis is a parasitic nematode that causes heartworm infection. It is a vector-borne disease transmitted by culicid mosquitoes and that mainly affects dogs and cats. Furthermore, *D. immitis* is a zoonotic parasite that causes pulmonary dirofilariosis in infected humans (McCall *et al*., 2008; Simón *et al*., 2012).

Heartworm is a worldwide distributed infection. Due to the humidity and temperature required by mosquito vectors, the disease is mainly present in tropical and subtropical areas. In Europe, *D. immitis* is endemic in southern countries (Genchi *et al*., 2009); however, heartworm is considered an emerging disease and is spreading toward eastern, central and northern Europe, affecting countries or areas previously considered free of the parasite (Morchón *et al*., 2012). Several factors have been reported as possible causes of this spread, such as climate change, the development of human activity for agricultural or urban uses in new areas, the growing movement of microfilaremic dogs throughout Europe or the introduction of new species of mosquitoes able to act as vectors. Also, infected wild animals can be reservoirs of the disease (Morchón *et al*., 2012; Simón *et al*., 2012).

Bulgaria is located in southeastern Europe. The country has mainly humid continental and oceanic climates (Kottek *et al*., 2006; Penin, 2007). Sofia is the capital of the country and is located in western Bulgaria, in the Sofia Valley, surrounded by the Balkan Mountains to the north, and Lyulin, Vitosha and Lozenska mountains to the

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southwest. The average altitude of the valley varies from 500 to 2290 meters and, unlike most of the European capitals, Sofia has no large rivers crossing it, but several small ones. Sofia has a humid continental climate, with cold, snowy winters and warm, sunny summers. The average annual temperature within the city is 10.6°C (Kottek *et al*., 2006; Penin, 2007). There are 1.5 million people living in the city and 1.7 million people living in its metropolitan area (Eurostat 2018a; 2018b).

Previous publications have shown presence of heartworm in Bulgaria. The first cases in dogs were documented in 1997 – 1999 and, since then, the presence of *D. immitis* has been described and studied in some regions of the country (Pantchev *et al*., 2015). Georgieva *et al*. (2001) reported a canine prevalence of 7.4 % in the Stara Zagora region. Between 2001 and 2006, microfilaremia was detected in 8.62 % of dogs from different Bulgarian districts (Kirkova *et al*., 2007) and there were 87 registered cases of canine heartworm in Plovdiv and surrounding regions (Kostadinov, 2007). Between 2012 and 2014, 34.3 % of the samples sent to a parasitology laboratory tested positive for *D. immitis* (Iliev *et al*., 2017) and a study determined 15 % of prevalence in 33 dogs from a shelter in Sofia in 2013 – 2014 (Radev *et al*., 2016). In 2015, a canine prevalence of 16.2 % in the Stara Zagora region was described by Pantchev *et al*. (Pantchev *et al*., 2015). The disease has also been observed and studied in wild carnivores, being described high prevalences in foxes and jackals (Kirkova *et al*., 2007; Mirchev *et al*., 2013; Panayotova-Pencheva *et al*., 2016).

These studies show the presence of heartworm in Bulgaria. However, there is a lack of current epidemiological data of canine *D. immitis* in Sofia. Therefore, the aim of this study was to obtain the prevalence and distribution of *Dirofilaria immitis* in dogs in the capital of Bulgaria and its metropolitan area.

Materials and Methods

The present study included 80 stray dogs from different parts of the city of Sofia and its metropolitan area. The animals were captured between December 2017 and February 2018 for routine neutering campaigns that take place in Sofia. A complete record was kept for each animal, including identification, age, sex, breed and location. The inclusion criteria were being dogs over 7 months of age and had not been treated with macrocyclic lactones.

Of the included dogs, 45 % were female and 55 % were male. The age ranged from 1 to 19 years old (mean: 7.1 years). Animals were further divided into 3 groups of age, from 1 to 4 years (n=25), from 5 to 9 years (n=29), and from 10 to 19 years (n=26). There were 60 mongrel dogs and 20 pure-bred dogs. According to the distribution, 38.75 % dogs were living in the city of Sofia while 61.25 % dogs were living in the metropolitan area.

Blood samples were collected from the cephalic vein of every studied animal and all the dogs were tested for circulating *D. immitis* antigens using a commercial immunochromatographic test kit (Uranotest Dirofilaria®, Urano Vet SL, Barcelona, Spain). The tests were performed according to the manufacturer's instructions. The data were analyzed using the SPSS Base 25.0 software for Windows. The descriptive analysis of the variables considered was carried out studying the proportions in the qualitative variables. The chi-square test was performed to compare proportions. In all the cases, the significance level was established at $p<0.05$.

Ethical Approval and/or Informed Consent

The design of the study was approved by the ethical committee of Veterinary Medicine Service of Las Palmas de Gran Canaria University and was carried out in accordance with the current European legislation on animal protection.

Results

The prevalence of *D. immitis* in the canine population in the studied area was 31.25 %. By sex, male dogs showed a higher incidence (36.4 %) compared to females (25 %). By breed, the most affected dogs were mixed-breed dogs (33.3 %) against purebred dogs (25 %). There were no statistically significant differences by sex or breed.

Positive dogs were found from 3 to 19 years, with an average age of 8.7 years. When age ranges were considered, the highest seroprevalences were found in the oldest part of the population, with 42.3 % of positives in dogs from 10 to 19 years old, followed by 37.9 % in dogs between 5 and 9 years. The lowest number of positive cases (12 %) was represented by dogs from 1 to 4 years ($p<0.05$).

The prevalence was higher in the metropolitan area of Sofia (34.7 %) than in the city of Sofia (25.8 %), although the differences were not statistically significant. The distribution of the positive cases of canine heartworm in the studied area can be seen in Figure 1.

Discussion

The present study reports the presence of heartworm in the stray dogs of Sofia and the metropolitan area. According to previous research, which reported prevalences from 7.4 % to 9.2 % in client-owned dogs in different regions of Bulgaria (Georgieva *et al*., 2001; Kirkova *et al*., 2007) and from 10 % to 15 % in stray dogs (Georgieva *et al*., 1992; 2001; Radev *et al*., 2016), the current results reported an increase in the prevalence of heartworm infections in the country. These results are consistent with studies that reported an increasing prevalence of *D. immitis* in Europe (Genchi *et al*., 2009; Morchón *et al*., 2012).

The institutions of Sofia are focused on decreasing the population of stray dogs, and there are no established control and prophylactic measures aiming to prevent the presence of different diseases, including heartworm. That is why the high prevalence reported in this study is probably influenced by the fact that all dogs were con-

Fig. 1. Map of the province of Sofia-city, where the distribution of the evaluated animals is shown. The urban part corresponding to the city of Sofia is shown as a darker color on the map. The blue dots correspond to negative dogs while the red squares correspond to heartworm-infected dogs (figures obtained and modified from a free media repository).

stantly exposed to mosquitoes and not receiving any prophylactic treatment. Probably, for the same reason heartworm infection was higher in the group of older animals. In 2015, it was estimated that 3844 (±10 %) stray dogs lived in the city, being one of the greatest problems of the capital (Ivanova & Gechev, 2015). Although the presence of microfilariae was not evaluated in the studied dogs, it is estimated that between 75 – 95 % of the infected dogs that do not receive chemoprophylaxis have microfilaremia (Stogdale, 1984), so these stray dogs may act as an important reservoir for heartworm, increasing the risk of infection of client-owned animals and humans living in the city.

The abundant vegetation and water reservoirs (green parks, artificial lakes and water ponds) present in the city of Sofia and in the metropolitan area may offer a perfect environment for reproduction and proliferation of the mosquito vectors which could favor the spread of the infection; furthermore, in the city of Sofia high

levels of urbanization can cause the phenomenon called Urban Heat Islands (Gago *et al*., 2013; Yang *et al*., 2016) which retains heat and increase the temperature inside the city. This indirectly influences the development of *D. immitis* larvae in mosquito vectors during the colder months and thus enlarges the transmission season (Arnfield, 2003). In Sofia, the average annual temperature of the urban area is 0.5°C higher than that of the peripheral area; moreover, in winter the temperature in the center of the capital is 1° – 1.5° higher than that of the rural regions (Kovachev, 2005).

Although the results of this work cannot be determined as definitive, given the small size of the sample studied, they are undoubtedly indicative of a widespread presence of the parasite in the city and indicative of the need for a broader study. In this regard, there is a lack of studies on heartworm in client-owned pets in Sofia and, according to the results obtained in this study, a high prevalence of *D. immitis* infection in these animals should be expected. This probably is due to the lack of knowledge of the general population about the disease, updated data on its epidemiology and prophylactic measures and campaigns aimed to avoid infection. Considering the growing trend of heartworm in different regions of the country, demonstrated by this and other recent studies (Pantchev *et al*., 2015; Panayotova-Pencheva *et al*., 2016; Iliev *et al*., 2017), a nationwide study to learn the current distribution of heartworm disease in Bulgaria in pets should be done.

There is a high risk of human dirofilariosis in areas of high canine prevalences (Simón *et al*., 2012; Cabrera *et al*., 2018). This can be observed in the increasing publication of cases of human infections by *D. repens* in Bulgaria (Harizanov *et al*., 2014; Velev *et al*., 2019). This is a zoonotic parasite that is increasing in Europe and the most frequent localizations are the eye region, subconjunctival or subcutaneous tissues in other body regions, forming nodules (Genchi and Kramer, 2017). Cases of pulmonary dirofilariosis by *D. immitis* have not yet been reported in the country; however, the apparent increase in the canine prevalence may increase the risk of infections in humans. Therefore, awareness of the disease should be promoted through the implementation of educational and prophylactic campaigns among veterinarians and owners. Also, competent authorities should be aware and alerted about the epidemiological situation in Sofia. Furthermore, being a zoonotic disease, health agencies and institutions of the country, as well as physicians, should consider the repercussions of this infection and include human dirofilariasis in the differential diagnosis of pulmonary nodules.

Conflict of Interest

Authors state no conflict of interest.

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Research Note

First report of *Pyelosomum cochlear* Looss 1899 (Digenea: Pronocephalidae) in a Hawksbill Turtle – *Eretmochelys imbricata L.* found in Brazilian Coast

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Summary

Pyelosomum cochlear Looss 1899 (Digenea: Pronocephalidae) is a parasite exclusive to sea turtles, having been described in the green turtle (*Chelonia mydas*) in Egypt, the USA, Panama, Costa Rica and Brazil as well as the olive ridley turtle (*Lepidochelys olivacea*) in Brazil. The present note describes the first occurrence of *P. cochlear* in a hawksbill turtle (*Eretmochelys imbricata*) found on the coast of Brazil.

Keywords: Brazil; Digenea; helmintofauna; *Eretmochelys imbricata*; Hawksbill Turtle; *Pyelosomum cochlear*; Pronocephalidae

Introduction

Looss (1899) erected the genus *Pyelosomum* (type species: *P. cochlear* Looss 1899) based on two specimens found in the urinary bladder of a green turtle (*Chelonia mydas* Linnaeus 1758) from Egypt. The parasite was later described in the same host in the United States (including from Puerto Rico) (Nigrelli, 1940, 1941; Dyer *et al*., 1991, 1995), Panama (Caballero, 1954), Costa Rica (Santoro *et al*., 2006) and Brazil (Werneck and Silva 2015) as well as in an olive ridley turtle (*Lepidochelys olivacea* Eschscholtz 1829) in Brazil (Werneck *et al*., 2015a). However, there are no previous reports of the occurrence of this parasite in the hawksbill turtle (*Eretmochelys imbricata* Linnaeus 1766).

Therefore, the present note describes the first occurrence of *P. cochlear* in a hawksbill turtle found on the coast of Brazil.

Materials and Methods

In December 2018, a hawksbill turtle was found dead after stranding on Brava Beach (23° 0'33.42"S / 44°29'3.00"W) in the city of Angra dos Reis, State of Rio de Janeiro, Brazil. The sea turtle measured 76.4 cm in curvilinear length and weighed 49.5 kg. During necropsy, the specimen was determined to be female, but the collection of samples for histopathological analysis was not possible due to the advanced state of decomposition of the carcass. However, the analysis of the urinary bladder revealed the presence of a single specimen of *P. cochlear* found in the mucosa of the organ.

The parasite was placed in saline solution, fixed in 70 % alcohol, stained with carmine and cleared with eugenol. The specimen was measured under a microscope (Nikon Eclipse 80i, Kurobane Nikon Co., Ltd., Otawara, Tochigi, Japan) with the aid of the NIS-Elements BR software and deposited in the Helminthological Collection of the Instituto Oswaldo Cruz (CHIOC number 38588) in the state of Rio de Janeiro, Brazil.

The identification of the parasite was based on the taxonomic genus key proposed by Blair (2005) and the phylogenetic study by Pérez-Ponce de León and Brooks (1995) as well as the de-

* – corresponding author

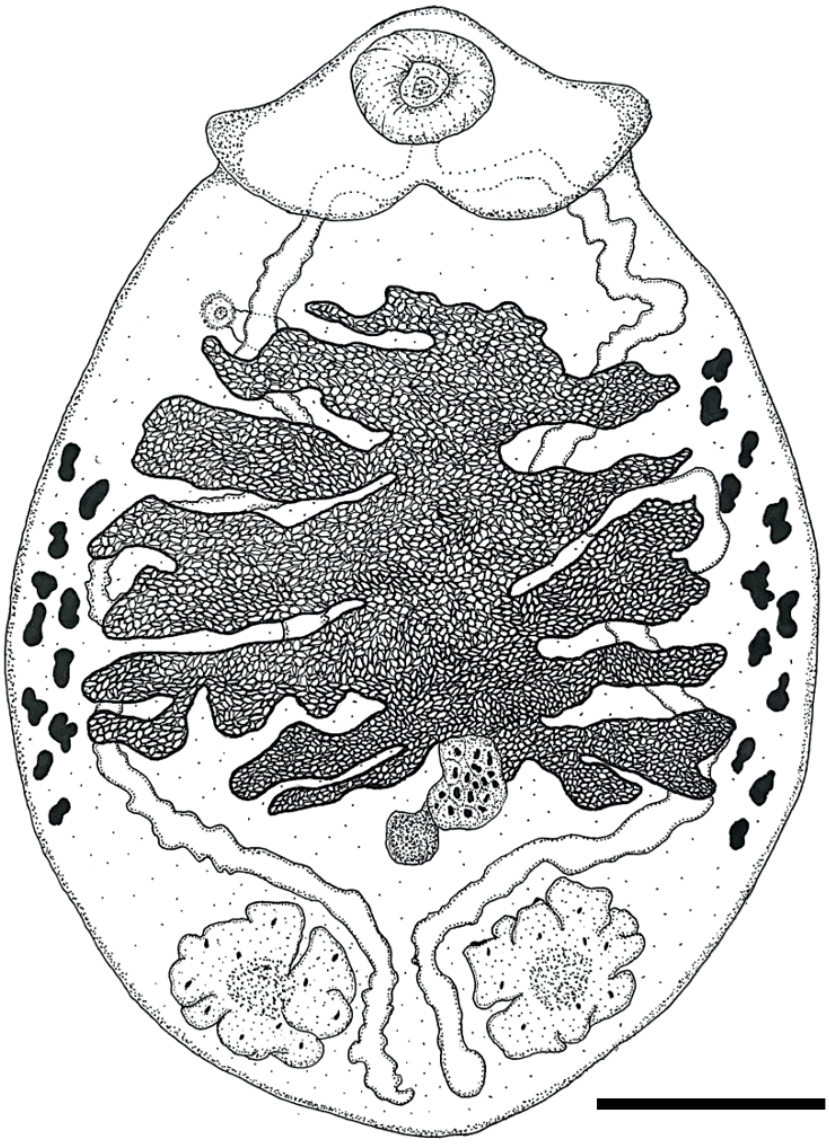

Fig. 1. *Pyelosomum cochlear* Looss 1899 (Digenea: Pronocephalidae) found in *Eretmochelys imbricata* Linnaeus 1766 (Testudines: Cheloniadae) from Brazil. Ventral view (scale bar 1 mm).

scription (Looss, 1899) and redescription (Caballero, 1954) of the species and other reports of the parasite (Nigrelli, 1940; Werneck *et al*., 2015a).

The following were the characteristics of the specimen (Fig. 1): anterior extremity tapered, with evident cephalic collar; posterior extremity rounded, measuring 4,282 µm in length by 3,409 µm in width; oral sucker subterminal, measuring 658 µm in length by 780 µm in width; esophagus short; ceca sinuous, near side of body, moving to mid region of body before testes, extending beyond the testes and terminating in fundus of cecum between testes near posterior extremity of body; cirrus sac partially covered by uterine loops, but clearly intercecal; vitellaria composed of two groups of follicles, extra-cecal, bordering side of body, beginning in anterior third near posterior extremity of cirrus sac and ending at ovary level, oval shape, right side with 11 follicles and left side with 12, some points surpass ceca; uterus begins at level of ovary and occupies practically entire medial (inter-cecal) area; uterine loops reach various points laterally to follicle area and anteriorly surpass cirrus sac area; testes in posterior region of body, extracecal, with slightly rounded and weakly lobed shape; right testicle measuring 852 µm in length by 807 µm in width; left testicle measuring 704 µm in length by 620 µm in width; ovary with irregular shape, anterior to Mehlis' gland, measuring 588 µm in length by 470 µm in width; Mehlis' gland measuring 374 µm in length by 223 µm in width; genital pore in anterior third of body at distance of 1209 µm from anterior extremity; eggs with polar processes (10 measured

without polar processes), ranging from 29 to 34 µm (mean: 31 µm) in length and from 13 to 18 µm (mean: 15 µm) in width. It was not possible to define the number of polar processes in each egg due to the quantity of eggs in the uterine loops.

Ethical Approval and/or Informed Consent

For this study formal consent is not required.

Discussion

Pyelosomum cochlear is a parasite exclusive to sea turtles and, according to the majority of reports, preferably occupies the urinary bladder of the host. The species was described based on two specimens found in the urinary bladder of green sea turtles found in Egypt (Looss, 1899).

Four decades later, Nigrelli (1940) cited the occurrence of *P. cochlear*, unfortunately, the author did not describe the number of individuals found, but presented morphometric data on length (0.5 to 2.5 mm), width (2.5 to 5 mm) and egg length (0.0763 to 0.253 mm) as well as some morphological characteristics. Analyzing 50 green sea turtles the following year, the author cited the occurrence of 16 species of parasites, including *P. cochlear* collected from the urinary bladder, but did not describe any morphometric/morphological characteristics or report the prevalence (Nigrelli, 1941).

Analyzing 12 green sea turtles found in Panama, Caballero (1954) redescribed *P. cochlear* based on a specimen found in the urinary bladder. Sometime later, Dyer *et al*. (1991) described the occurrence of *P. cochlear* in the cloaca of a green turtle found in Puerto Rico.

Dailey *et al*. (1992) reported the occurrence of immature individuals of *P. cochlear* in the urinary bladder of a green sea turtle found in Oahu, Hawaii. Dyer *et al*. (1995) reported the occurrence of two specimens of *P. cochlear* in two of a total of four green sea turtles analyzed in Puerto Rico.

Santoro *et al*. (2006) analyzing 40 adult green turtles and described the occurrence of *P. cochlear* in 57.5 % of the hosts, with a mean intensity of 1.8 ± 1.1 (range: 1 to 4). In an analysis of 136 juvenile green turtles from the coast of Brazil, *P. cochlear* was found in only one host (Werneck and Silva, 2015). More recently, Werneck *et al*. (2015a) reported the occurrence of a specimen of *P. cochlear* in the urinary bladder of an olive ridley turtle in southern Brazil.

The specimen analyzed in the present study was consistent with the original description by Looss (1899) and redescription by Caballero (1954) and had the majority of characteristics described by Pérez-Ponce de León and Brooks (1995), with the presence of uterine loops exceeding the limits of the cirrus sac (i.e., autapomorphy found only in *P. cochlear*). However, it was not possible to determine/describe the filament number in each egg, development of the prostatic complex or degree of glandulation of the metraterm due to the large number of eggs in the uterine loops.

The morphometric analysis revealed that the oral sucker, right testicle, length of the left testicle, length of Mehlis' gland and ovary dimensions were larger than previously published data and the egg dimensions were smaller than previously published data (Looss, 1899; Nigrelli, 1940; Caballero, 1954; Werneck *et al*., 2015a). Such findings likely correspond merely to individual variations of the specimens

The helminth fauna of the hawksbill turtle correspond to approximately 60 species distributed among 11 families (see Dyer *et al*., 1995; Greiner, 2013; Santoro *et al*., 2015; Werneck *et al*., 2014, 2015b, 2015c). The present study adds *P. cochlear* to this list.

Conflict of Interest

Authors state no conflict of interest.

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Research Note

Morphological characterization of *Dujardinascaris* spp. (Nematoda: Anisakidae) from the striped red mullet *Mullus surmuletus* in the Mediterranean Sea

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Article info

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Summary

The striped red mullet *Mullus surmuletus* (Linnaeus, 1758) (Perciformis: Mullidae) has a high commercial value and therewithal is a common demersal fish of the Mediterranean Sea, therefore studying the helminth parasites of this fish is required. Anisakids nematodes are common parasites of animals including human causing economic losses and different parasitic diseases. During the present study, the nematode *Dujardinascaris* spp. (Anisakidae) was described from the body cavity and small intestine of *Mullus surmuletus* in the Alexandria, Mediterranean Sea, Egypt as new host and new geographical record. Forty-five (37.5%) fish out of (120) were found infected by the parasite. The morphological features of the collected nematode were investigated by both light and scanning electron microscopy. The study revealed that the specimens were characterized from other species of the genus by the presence of two large lateral pouches attached to each cephalic lip, the different large-sized papillae on the cephalic region and on the dorsal surface of the nematode cuticle.
Keywords: light microscopy; *Mullus surmuletus*; Nematode; scanning electron microscopy (SEM)

Introduction

The striped red mullet *Mullus surmuletus* (Linnaeus, 1758) (Perciformis: Mullidae) is one of the most abundant and widely distributed fish in the sublitoral zone along the Eastern Atlantic, from the North Sea to the northern part of West Africa and the Mediterranean Sea (Klimpel *et al*., 2008). Several authors studied the helminths of *M. surmuletus* such as Figus *et al*. (2005) who identified 18 species of helminths with total infection rate (65.5 %) in Italy. Ferrer *et al*. (2005) from the Spanish Western Mediterranean Coast. Bayoumy *et al*. (2008) from Syrt Coast, Libya. Also, Klimpel *et al*. (2008) in the North Sea and Mediterranean Sea. *Mullus surmuletus* occurs on broken and rough grounds in less than 100 m water depth. It is highly infected in the M. Sea hosting about 28 different species of parasites (Bartoli & Bray, 1996; Ferrer *et al*., 2005). Mediterranean Sea is characterized by an unusual high species diversity for a temperate sea. It contains around 7 % of the total global marine fish species with a wide range of both tropical and temperate species. Nematodes considered as one of the largest and most diverse group of helminth parasites infect marine, freshwater and even brackish water fish (Klimpel *et al*., 2011; Morsy *et al*., 2012). Their infections cause great morbidity in humans and animals. Numerous cases of human anisakiosis especially of genera Anisakis and Pseudoterranova occur due to the consumption of undercooked fish (Chaligiannis *et al*., 2012; Guardone *et al*., 2018). Food-borne zoonosis in aquatic animals are most linked to anisakid nematodes. They are common parasites of mammals, reptiles, fish and fish eating birds with a worldwide distribution causing diseases and important economic losses (Dadar *et al*., 2016). Among this group members of

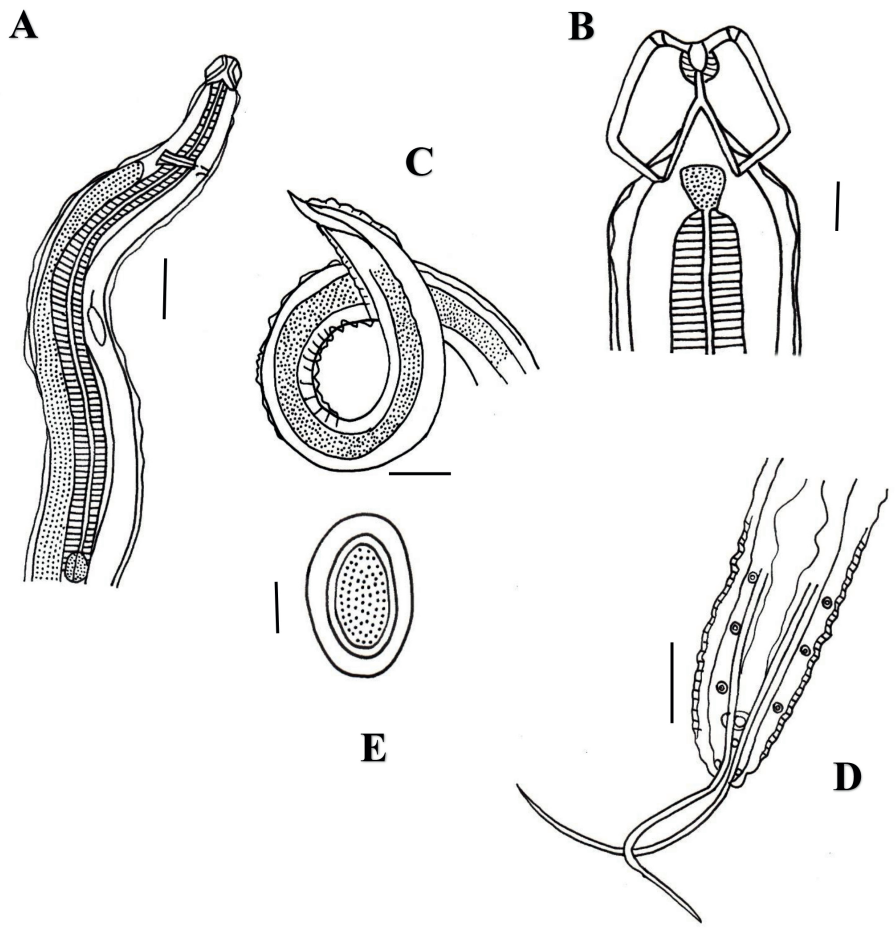

Fig. 1 (A – E): *Dujardinascaris* spp. from Red Mullet *Mullus surmuletus* in Mediterranean Sea. A: Anterior end of male dorsal view, B: cephalic region of male, C: The posterior end of female subventral view, D: The posterior end of male ventral view, E: Egg. Scale bars: A,C 0.2; B 200 µm; D 0.3; E 50 µm.

the genera *Anisakis* Dujardin, 1845, *Pseudoterranova* Mozgovoi, 1951 and *Contracaecum* Railliet and Henry, 1912 may be a problem for the commercial fishing industry (Amor *et al*., 2011; Klimpel & Palm, 2011). Based on light and scanning electron microscope studies, the present nematode was described from the striped red mullet *Mullus surmuletus* in the Alexandria, Mediterranean Sea, Egypt. It represents the first record in the Mediterranean and the first time for recording from the host *M. surmuletus*. The subfamily Heterocheilinae Railliet and Henry, 1912 have species parasitize mainly adult vertebrates such as crocodilians, tortoises, sirenians and some other fish species (Sprent 1990). The nematode genus *Dujardinascaris* was established by Baylis, 1947. It includes about 20 species most of them parasitize lizards and crocodiles but a few number infecting fish (Masova *et al*., 2014).

Materials and Methods

A total of 120 striped red mullet *Mullus surmuletus* were obtained from local fishermen operating on Alexandria coast, Mediterranean Sea, Egypt. The hosts were transported to the laboratory for their identification according to (Burgees *et al*., 2000; Schultz 2003). They were dissected, the abdominal cavities were inspected and internal organs were removed, separated in petri dishes and washed very well by saline solution (0.7 %). The intestine was opened carefully searching for helminth parasites and its content was examined using a dissecting stereomicroscope. The collected nematodes were isolated, counted, their sites were recorded and then they washed several times in physiological solution. Seventy-four female and thirty-eight male of the genus *Dujardinascaris* were obtained. The specimens were fixed by 7 % formalin for about 12 h. for morphological identification, the fixative was gradually replaced by lactophenol solution, cleared in few drops of lactophenol then mounted by DPX. Photomicrographs were taken using a microscope supplied with digital camera. Drawings were made by Camera Lucida. All measurements were taken in millimeters unless otherwise stated. The nematode specimens were identified

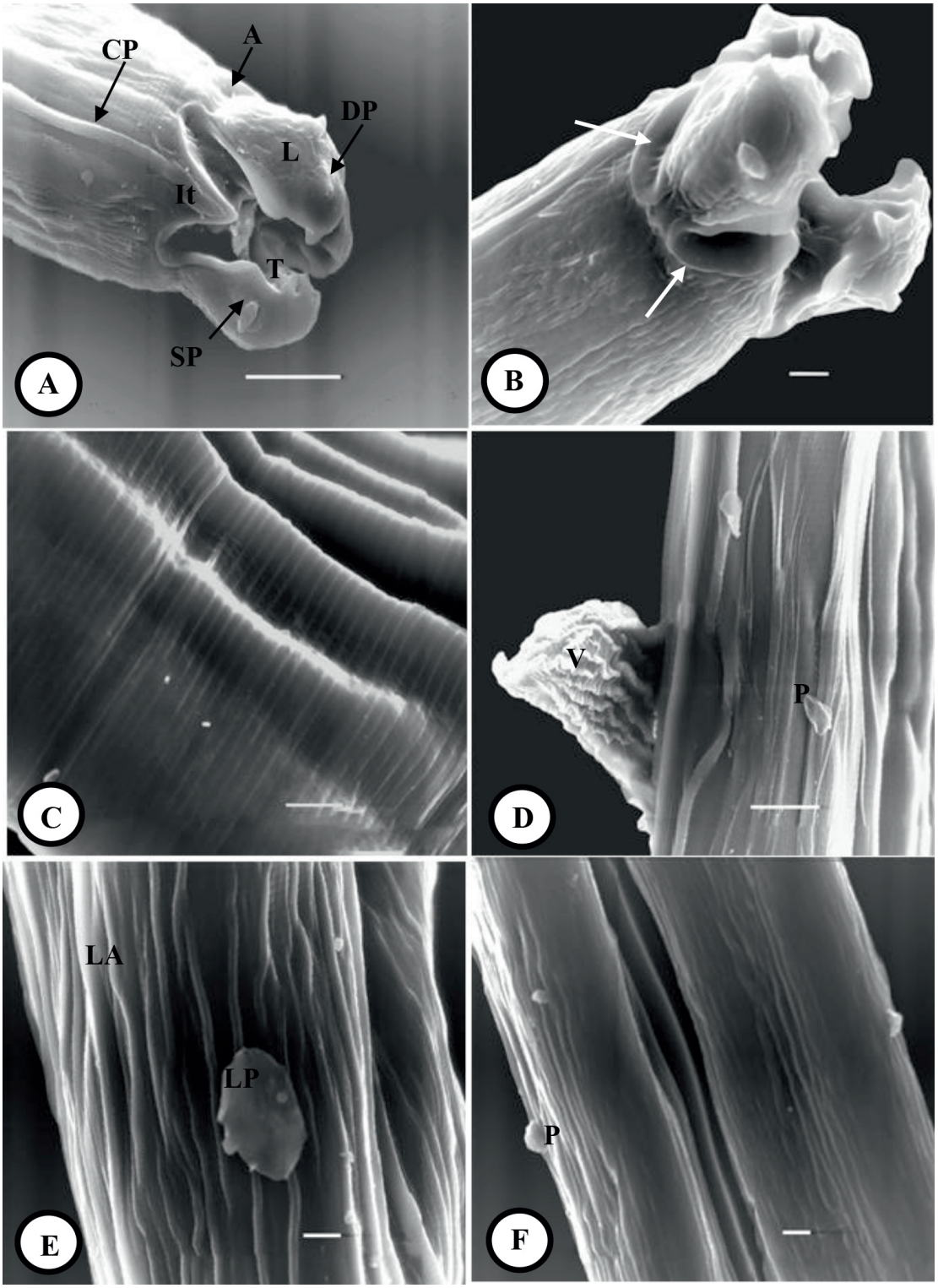

Fig. 2 (A – F): Scanning Electron Microscope of *Dujardinascaris* spp. (A): The cephalic region of the worm with interlocked processes, A amphid, L lip, It interlabium, T teeth, DP double papillae, SP single papilla, CP cuticular prolongation. (B): the anterior end showing the cephalic lateral pouches (arrows). (C): The middle region of nematode body showing the cuticular transverse striations. (D): The vulvar region with protruded vagina V. (E): The dorsal view of somatic region showing the longitudinal annulation LA, large rounded-like papilla LP. (F): The post-equatorial part of nematode body showing papillae P.
Scale bars: A,D,H,I,K,L = 50 µm, B,C,EF,G,J = 10 µm.

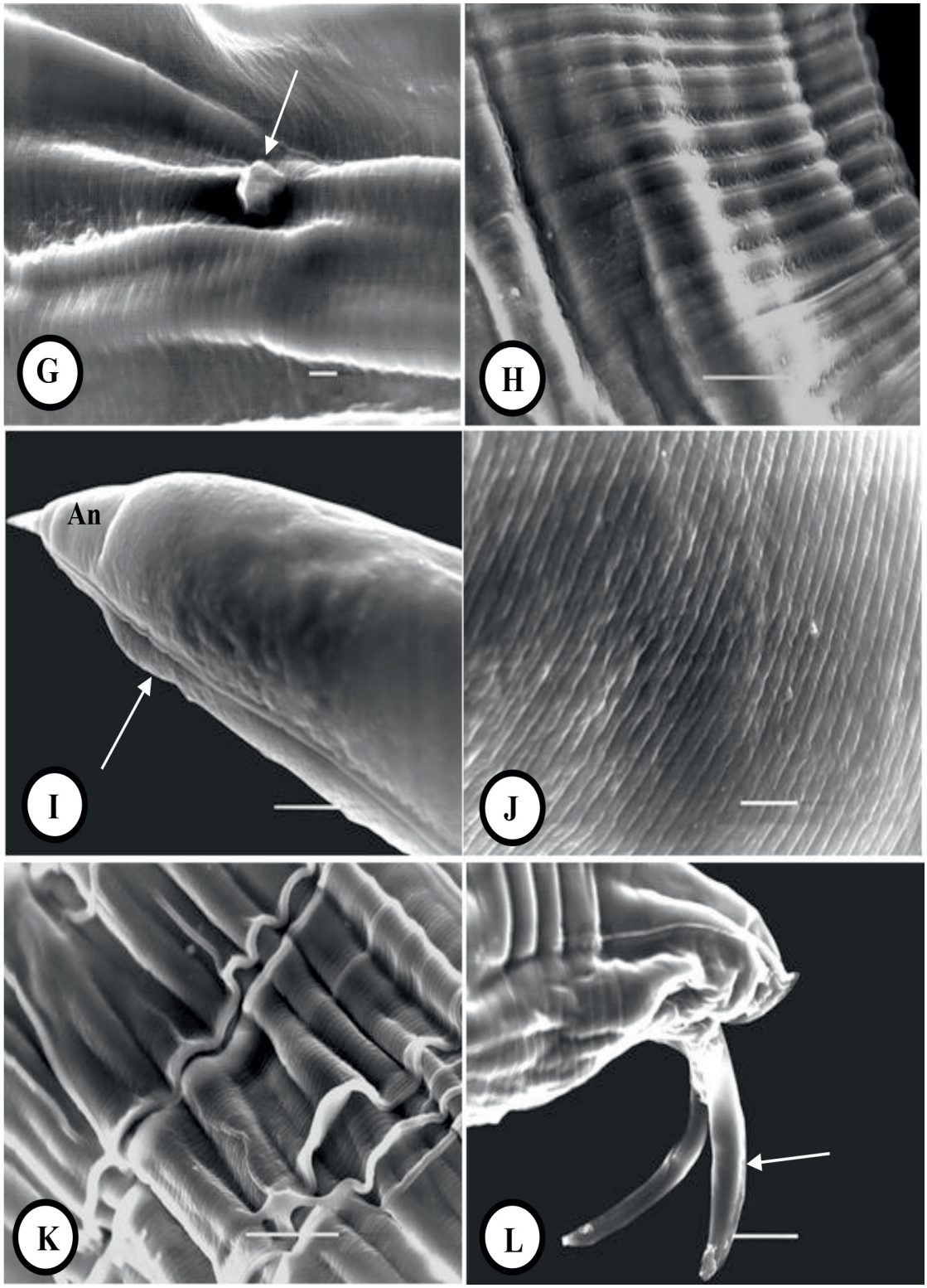

Fig. 2 (G – M): Scanning Electron Microscope of *Dujardinascaris* spp. (G): The ventral surface of the worm at cuticular region showing buttom-like papilla (arrow). (H): The cuticle at the posterior region of female worm showing the transverse striations. (I): The posterior end of female showing anus An & the caudal alae (arrows). (J): The cuticle at the anal region showing the transverse striations. (K): The posterior end of male showing the cuticular transverse striations. (L): The posterior end of male showing spicules (arrows). Scale bars: A,D,H,I,K,L = 50 µm, B,C,EF,G,J = 10 µm.

according to (Yamaguti 1961; Anderson *et al*., 2009). The techniques used in the present study help in the observation as well as the identification of parasites. For scanning electron microscopy, the nematodes were fixed in 2 % glutaraldehyde in 0.1 M. sodium cacodylate buffer (PH 7.2), dehydrated in ethyl alcohol, critical point dried, mounted on stubs carefully and coated with gold then they were examined and photographed at varying magnifications using a JOEL JSM-5400LV scanning electron microscope at an accelerating voltage of 15 KV at Electron Microscopic Lab of the Atomic Energy Agency, Nasr City, Cairo, Egypt.

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. For this study formal consent was not required.

Results

Dujardinascaris spp.
Family: Anisakidae Railliet & Henry, 1912
Subfamily: Heterochilinae Railliet & Henry, 1912
Type-host: The striped red mullet *Mullus surmuletus* Linnaeus, 1758
Site in host: Intestine and body cavity
Locality: Depth ranges from 5 – 60 at tropical level 3.5, Mediterranean Sea, Alexandria, Egypt.
Prevalence and intensity: 45 infected out of 120 examined fish with prevalence (37.5 %), 1 – 4 specimens per fish.
Deposition of voucher specimens: Parasite specimens are deposited in the helminthes collection in the Zoology Department, Faculty of Education, Ain Shams University.

Description: light and scanning electron microscopy
It is medium to large in size, white in color, females are longer and more pointed posteriorly than males. Cuticle is transversally striated. Cervical and caudal alae present, the cephalic region has three large lips with distinct interlocked processes. The dorsal lip has two convex processes for articulating with the ventrolateral lips. Each lip is supported at its internal edge with row of 3 – 4 small pointed teeth. Interlabia large and well developed. Two lateral amphids, double cephalic sensory papillae on the dorsal lips and a single one on the sub ventral lips were found (Fig. 2 A&B). A cervical ala was extended from the mouth opening along the nematode body. Dentigerous ridges absent. In addition, SEM revealed a pair of large symmetrical wide pouches located at the base of each lip (Fig.2B). A morphological variation of the transvers striations was observed along the whole body worm (Fig.2 C,E,J,K). Different shapes of papillae were found on the vulvar and post-vulvar region of the nematode body (Fig.2 D&E). Also, a large flattened-like papilla was observed on the middle part of dorsal body worm (Fig.2G). A pair of sensory lateral papillae were noted on the cuticle of somatic region that follow the vulvar region (Fig.2F). Esophagus is muscular, long and a small rounded ventriculus is present (Fig. 1A). Nerve ring encircling esophagus at about 30 % of its length. Excretory pore is located approximately at the same level of the nerve ring. Vulva is pre-equatorial in the first third of the body. The cuticle of the present nematode exhibited distinctive transverse striations at various areas along the nematode body. The dorsal surface that follow the cephalic region has an irregular longitudinal annulations interrupted by papillae-like structures while the region that follow the vulva has regular transverse striations. The annuli at the last third of male specimen has a distinct pattern as the cuticular annuli are parallel to each other and subdivided transversally. The cuticular surface of the last part of female body is characterized by narrowed transverse striations separated by fine grooves (Fig.2 C,E,H,J,&K). Male: (Based on six specimens): Body 10 – 14.2 long and 0.33 – 0.47 wide. Lips 0.06 – 0.085 long, interlabia 0.033 – 0.041 long. Esophagus 1.21 – 1.66 long with 0.062 – 0.071 wide representing (10 %) of body length. Nerve ring and excretory pore at 0.35 – 0.38 and 0.37 – 0.39 respectively from the anterior extremity. Intestinal caecum 0.71 – 1.33 long representing (8.5 %) of body length. Spicules are long, slender, equal in size measure 2.23 – 3.45 µm. long. Gubernaculum is present 0.08 – 0.11 long. Tail 0.11 – 0.17. Caudal papillae are observed by light microscope in the form of four pairs: three pairs of precloacal, one pair of post cloacal papillae and one median cloacal papilla. A pair of lateral phasmids was observed (Figs.1D). Female: (Based on six specimens): Body 13 – 22.5 long and 0.18 – 0.24 wide. Lips 0.09 – 0.15 long. Interlabia 0.05 – 0.062 long. Esophagus 1.67 – 2.1 long and 0.07 – 0.082 wide representing (12 %) of body length. Nerve ring and excretory pore located at 0.41 – 0.52 and 0.46 – 0.57 respectively from anterior extremity. Intestinal caecum 1.11 – 1.43 long. Vulva is post-equatorial (0.093 – 1.15) from the anterior end. The muscular vagina was extruded outside from vulva on the ventrolateral surface at the first third of nematode body (Fig.2D). Tail 0.34 – 0.52 long, it is ended by small caudal spike (Fig.1C). The distal end of female is provided by an elevating transverse outgrowth (Fig.2I). The cuticle of last third of female body has transverse striations that separated by internal fine grooves. The cuticle surrounding the anus region characterized by the absence of annulations. Eggs have smooth surface, oval shaped and measure 52 – 90 x 40 – 78 µm (Fig.1E).

Discussion

Dujardinascaris (Table 1) parasite currently includes 20 nominal species recorded from different hosts of lizards and fishes (Li *et al*., 2015). It was firstly established by Baylis, 1947 from crocodiles. Previous studies indicated that most species of that genus were isolated from crocodiles while few of them parasitize fishes (Sprent, 1990, Li *et al*., 2015). Yamaguti, 1941 has been listed 13 species in genus *Dujardinascaris*, two of them have been reported from fishes and 11 species from reptiles (Morsy *et al*., 2013).

Table 1. Morphometric comparison of some species of nematode *Dujardinascaris* from fishes with the present species. (The data for the previous described species are taken from the original descriptions).

Characters	*D. melapteruri*	*D. mujibi*	*D. mormyropsis*	*Dujardinascaris* spp.
Length ♂	14.8	17 – 24	11.5 – 15.33	10 – 14.2
♀	12.3 – 35.0	23 – 38	26 – 43.22	13 – 22.5
Width ♂	0.3	0.25 – 0.65	0.38 – 0.54	0.33 – 0.47
♀	0.25 – 0.68	0.76 – 0.84	0.089 – 0.1	0.18 – 0.34
Lips L. ♂	70 µm	–	75 – 90 µm	70 – 98 µm
♀	50 – 110 µm		135 – 159 µm	50 – 90 µm
Interlabia L. ♂	–	–	30 – 45 µm	33 – 41 µm
♀			78 – 96 µm	40 – 60 µm
Esophagus L. ♂	1.7	2.25 – 2.75	1.32 – 1.84	1.21 – 1.66
♀	1.3 – 2.6	4.45 – 5.84	2.2 – 3.07	1.67 – 2.1
Esophagus W. ♂	–	0.11 – 0.15	0.08 – 0.11	0.062 – 0.071
♀		0.15 – 0.18	0.09 – 0.12	0.07 – 0.082
Nerve ring * ♂	0.35	–	0.42 – 0.56	0.35 – 0.37
♀	0.27 – 0.41		0.58 – 0.77	0.41 – 0.52
Excretory pore *♂	0.36	–	0.42 – 0.56	0.36 – 0.39
♀	0.4		0.58 – 0.81	0.46 – 0.57
Intestinal canal ♂	1.1	–	0.68 – 1.11	0.71 – 1.33
♀	0.77 – 2		1.19 – 1.73	1.11 – 1.43
Tail L.	0.08 0.15 – 0.41	–	0.12 – 0.17	0.11 – 0.17
Spicule L.	1.4 µm	1.13 – 1.34 µm	1.7 – 1.81 µm	2.23 – 3.45 µm
Gubernaculum L.	–	–	0.186 – 0.2	0.08 – 0.11
Vulva *	–	–	9.13 – 15.92	0.093 – 1.15
Egg L.	–	–	0.06 – 0.09	0.052 – 0.09
Egg W.	–	–	0.052 – 0.072	0.04 – 0.078
Caudal alae	Absent	Absent	Absent	present
Host species	*Malapterurus electricus*	*Pagrus pagrus*	*Mormyrops anguilloides*	*Mullus surmuletus*
Locality	Khartoum, Sudan	Red Sea, Egypt	Central Africa	Mediterranean Sea, Egypt
Author	Sprent, (1990)	Morsy *et al*. (2013)	Moravec & Jirku, (2014)	Present study (2019)

Abbreviations: L.=length; W.=width; ♂= male; ♀=female; * From anterior extremity

Sprent (1977) also listed 12 species of that genus from crocodilians worldwide. Machida *et al*. (1992) described *D. philippinensis* from *Crocodylus porosus* in Philippines. Sprent *et al*. (1998) described five species of the present genus from crocodiles in Australia and New Guinea; *D. patterae*, *D. blairi, D. harrisae, D. westonae* and *D. angusae*. Moravec *et al*. (2014) reported *D. madagascariensis* from *C. niloticus*. Li *et al*. (2015) discovered *D. gigantea* from *Alligator sinesis* in China. Species of genus *Dujardinascaris* that reported from fishes included *D. cybii* by Lakshmi and Sudha (2000) from intestine of *Mugil cephalus* as new host in India. It was characterized by slender pointed tail with cuticular ring-shaped striations and absence of caudal alae. *D. mujibii* described by Morsy *et al*. (2013) from the intestine of *P. pagrus* in the Red Sea. It is similar to the present species in the shape of lips as each one has 3 – 4 small pointed teeth. It differs from it in the situation of the vulva in the first third of the body in the present species (*vs* post-equatorial), in the body measurements and in the absence of caudal alae in *D. mujibii*. Also, *D. mormyropsis* described by Moravec and Jirku (2014) from stomach of *Mormyrops anguilloides* in central Africa. It was mainly distinguished from the present species by the number of teeth (10) that oriented from the lips, the presence of dentigerous ridges on lips, in the absence of cervical and caudal alae and finally in the measurements of caecum and esophagus length. From the other hand, the present species agrees with *D. mormy-*

ropsis in the distribution of papillae on the cephalic region. *Dujardinascaris malapteruri* Baylis, 1947 syn. *D. graberi* Troncy, 1969 is the only known valid species of the present genus parasitizing African freshwater fishes as adults (Moravec & Jirku, 2014), it was described by Sprent (1990) in Chad from *Malapterurus electricus*. He distinguished that species by the anterior prolongation of the dorsal lip, ten pairs of caudal papillae on male tail. Bannai *et al*. (2016) studied *D. sphyraenaii* and its pathological effects on fish *Psettodes erumei* in Arabian Gulf. Sood (1989) had proposed a key for species of *Dujardinascaris* Baylis 1947 from fishes in south Asia included five species; *D. magna* Khan & Begum, 1971; *D. ritai* Zaidi & Khan, 1975 from *Rita rita* in Lahore; *D. quadrii* Zubari & Farooq, 1976; *D. sciaena* Bilqees *et al*. 1977 from *Sciaena* sp. in Karachi Coast and *D. cybii* Arya & Johnson, 1978 from *Cybium guttatum* in India. Moravec and Jirku (2017) redescribed *D. malapteruri* from fish *Malapterurus monsembeensis* in Congo River, they studied it by scanning electron microscope and characterized the species by the presence of dentigerous ridges on lips and absence of caudal alae. Although the lizards and crocodiles are more exposed to the infection by the present nematode, it was clear that genus *Dujardinascaris* had the ability to infect and adapt in a variety of fish hosts and may infect more and more of fish species. The present finding of *Dujardinascaris* from *Mullus surmuletus* fish represents a new host record and a new geographical record from Mediterranean Sea. Moravec and Jirku (2014) suggested that freshwater fish play a role of the intermediate hosts, in which the third stage larvae of the worm may occur. The present genus rarely infects marine water fishes, this may be due to different reasons such as the distribution of the final and intermediate hosts, thus these parasites can overcome wide distances if they infect migratory species. Parasites of fishes have been used as biological indicators for ecology of hosts and their migration in successful way (Klimpel *et al*., 2008). Scanning electron microscope of the cephalic capsule and distal end of the present species ensures that it is belonging to genus *Dujardinascaris*. The presence of three large cephalic lips each one is supported by a number of small chitinous pointed teeth, this is a taxonomic character for the genera of subfamily Heterochilinae and consequently for genus identification. The number of teeth differs through different species of genus *Dujardinascaris*. The number of teeth vary from three to ten in nematode species that infect fish hosts while in case of species that infecting lizards and crocodiles the number of teeth ranged from 10 – 20 (examples: *D. philippinensis* from *Crocodylus porosus* Machida *et al*., 1992 and *D. gigantea* from *Alligator sinensis* Li *et al*., 2015). Teeth seem to have a penetration function in the intestinal wall of the host (Bayoumy *et al*., 2008). The cuticle of the present nematode exhibited distinctive transverse striations at various areas along the nematode body. Such cuticle striations were previously recorded in many species of nematode fishes (*Hysterothylacium alatum* Moravec and Justine, 2015; *Eustrongylidae exciscus* Gupta, 2019). The usefulness of cuticle morphology for the identification of Anisakids and their developmental stages (Molina-Fernandez *et al*., 2018). The pair of phasmids at the caudal end of male considered to have glandular and sensory function. The surface topography of parasites is an important character to understand the intricate relationship between them and their hosts. In all respects the present described nematode is similar to great extant to the specimens that described by Morsy *et al*. (2013) except some differences included the distribution of papillae on the cephalic region, the large pouches that are attached to lips, also, the distribution of different shapes of papillae.

The helminth parasites of the striped red mullet *Mullus surmuletus* have been studied by several parasitologists due to its economic importance. Le Pommelet *et al*. (1998) listed a high diversity of trematodes infecting Mullets from Western Mediterranean and Adriatic Sea. Ferrer *et al*. (2005) in the Spanish Western Mediterranean Coast. Bayoumy *et al*. (2008) studied the ultrastructure of four species of helminth parasites infecting *M. surmuletus* in Syrt coast, Libya with parasitic prevalence (67.6 %). Also, Klimpel *et al*. (2008) demonstrated that the mullets of the North Sea are more infected with helminthes than those from the Mediterranean Sea. Hassani *et al*. (2015) carried out an epidemiological survey of helminths infecting *M. surmuletus* in Algeria, Western Mediterranean, they recorded 14 species of helminths included trematodes, nematode, cestode and acanthocephalan. These differences in the parasite fauna composition of Mullets may be regarded to historical and geographical reasons (Hassani *et al*., 2015).

Conflict of Interest Statement:

Author declare that she has no conflict of interest pertaining to this submission.

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Research Note

Occurrence of *Anisakis* and *Hysterothylacium* nematodes in Atlantic chub mackerels from Libyan coasts

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Summary

The occurrence of zoonotic parasitic nematodes in Atlantic chub mackerels (*Scomber colias* syn. *Scomber japonicus*) from Libyan waters was investigated, using epizootiological estimations and molecular specific characterization of larvae. Nematodes belonging to *Anisakis* spp., the main etiological agent of anisakiasis in Mediterranean waters, and to *Hysterothylacium* spp. so far considered not pathogenic to humans, were detected. Prevalence values were generally high in visceral cavities (over 40 % for both parasites) while were low for *Anisakis* (around 1 %) and null for *Hysterothylacium* in muscles. Moreover, the level of infections was associated with seasons, a feature potentially useful to plan fishing captures and to elaborate risk mitigation strategies for anisakiasis. Species molecular identification performed on a subsample described the presence of *Hysterothylacium aduncum* as the predominant species, along with *Anisakis pegreffii* and the hybrids (*A. pegreffii* and *A. simplex* sensu stricto), thus posing a concrete zoonotic risk following the consumption of such fish species as a raw preparation.

Keywords: Atlantic chub mackerel; *Anisakis pegreffii*; *Hysterothylacium aduncum*; PCR-RFLP; seasonality; consumers' safety

Introduction

An adequate intake of fishes would ensure around 50 % of daily requirement of animal protein. Actually, it accounts for the 16 % of the animal proteins consumed by the world's population, and many marine products, such as mackerels, sardines and anchovies are widely consumed in the Mediterranean coastal regions for the high amount of fatty acids and omega-3, which are of significant nutritional value. The last available data on fish consumption reported 20.2 kg in 2015 up to 20.5 kg in 2017 (FAO 2018).

The Atlantic chub mackerel *Scomber colias* Gmelin, 1789 (Family Scombridae) is a pelagic fish largely used along with anchovies and sardines, both for human and animal nutrition. The taxonomic assignment of members of this fish species in the Mediterranean has been intensively studied in recent years, following the evidence that the Pacific and the Atlantic/Mediterranean populations are significantly differentiated and may represent two distinct species (Infante *et al*., 2007). According to these authors, the term *Scomber japonicus* should be retained for chub mackerels from the Pacific and the term *S. colias* for the Atlantic and the Mediterranean. In general, mackerels represent a large amount of all fishes captured worldwide (FAO 2007) and the most caught species in Portugal in 2016 (>26.000t) (DGRM 2017), ranking among the most valuable fish species in Europe (Levsen *et al*., 2018). Atlantic chub mackerels are frequently infected by anisakid nematodes (Costa *et al*., 2003; 2011; Debenedetti *et al*., 2019),

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parasites with a marine life cycle maintained by a prey-predator trophic web among intermediate, paratenic and definitive hosts. The occurrence of nematodes in marine products causes economic losses and medical problems, as larval nematodes belonging to the family Anisakidae may cause a fish-borne zoonotic disease in humans known as anisakidosis, while those belonging to the family Raphidascarididae are commonly considered not zoonotic or of negligible concern for human health (Klimpel & Palm, 2011). In Mediterranean countries, the species *Anisakis pegreffii* is the main etiological agent of anisakiasis, as it is widely spread in paratenic and definitive hosts of Mediterranean waters; similarly, *Hysterothylacium aduncum* is one of the most frequently Raphidascaridid recovered in teleosts, representing mainly an aesthetic problem, potentially constraining fish marketability.

Following the European Authority for Food Safety encouragement to continuous research on aspects related to anisakids and fishes intended for human consumption (EFSA 2010), the aim of the present study was to investigate the presence of *Anisakis* spp. and *Hysterothylacium* spp. in specimens of *Scomber colias* caught off Libyan waters. Although the occurrence of anisakid nematodes in fishes of the western part of the Mediterranean Sea has been largely studied, information on the southern-central part of the Mediterranean are still scarce and worthy of investigation. Only few studies are available on anisakids infecting marine fishes from Libyan coasts but not involving mackerels among the sampled fish species, and in most cases limiting the identification of nematodes at genus level (Sharif & Negm-Eldin, 2013; Kassem & Bowashi, 2015; Eissa *et al*., 2018). An additional aim was to characterize a subsample of parasitic nematodes at molecular level to identify larvae at species level and to indirectly infer the risk related to fish consumption.

Material and Methods

Sampling and infection parameters

A total of 360 individuals of *Scomber colias,* measuring 20.1 – 26.3 cm and weighting 70.3 – 158.2 g, were purchased from the fish market in Tripoli, Libya, from September 2013 to August 2014, sampling 90 specimens for each season. Stored in ice, the fish were directly transferred to the Laboratory of Quality and Diseases of Marine Organisms at the Marine Biology Research Center. Visceral cavities were visually inspected and the remaining parts were filleted. All material was checked for the presence of larval nematodes using a stereomicroscope and with light candling method according to EFSA (2010).

The recovered nematodes, excluding those randomly selected for molecular identification, were fixed, clarified and preserved as previously described (Eiras *et al*. 2006; Lasee 2004; Navone *et al*. 1998 and Roberts 1989). The larvae were morphologically identified at genus level according to available diagnostic keys (Berland 1961; Davey 1971).

Epizootiological parameters as prevalence, mean abundance and intensity of infection as defined by Bush *et al*. (1997), and ecologically relevant parameter as aggregation index, were calculated using Quantitative Parasitology (Reiczigel *et al*., 2013). Parameters were calculated as indices of overall infections in relation to parasitic genus, to seasons (trimesters) and to anatomic site of larvae recovery (body cavity vs fillets). Statistical significant differences among prevalence values, with regard to seasons, were evaluated by using the chi-square test or Fisher's test.

Species identification with molecular diagnostic keys

A subsample of 100 larvae, randomly selected among different capture batches, were characterized at genetic level using a molecular approach based on PCR–RFLP of the nuclear ribosomal internal transcribed spacer (ITS) region, since it is informative for taxonomic/diagnostic purposes for the genera *Anisakis* and *Hysterothylacium* (D'Amelio *et al*., 2000; De Liberato *et al*., 2013). In details: genomic DNA was isolated from entire larvae using the Wizard Genomic DNA purification kit (Promega, Madison, WI), according to the manufacturer's protocol. In brief, after removing the anterior and posterior ends for morphological studies, the remaining part of individual nematodes were each placed in 600 µl of a mixture containing 0.5 Methylenediaminetetra-acetic acid (EDTA) plus Nucleilysis solution and then crushed employing a sterile pestle. Proteinase K (17.5 µl at 20 mg/ml) was added to each tube, which was incubated at 55°C for 3 h. RNase solution (3 µl at 4 mg/ml) was added, and the tubes were incubated at 37°C for 30 min. Subsequently, protein precipitation solution (200 µl) was added, the tubes were vortexed and chilled on ice for 5 min, and the DNA was precipitated with ethanol. Each DNA pellet was air-dried for 20 min and dissolved in 100 µl of DNA rehydration solution.

The entire ITS region (ITS-1, 5.8S, ITS-2), of around 1000 base pairs, was amplified using 20ng of template DNA, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl2 (Bioline), 40 mM of nucleotide mix (Promega), 50 pmol/µl of the primer forward NC5 (5-GTAGGTGAACCTGCGGAAGGATCAT-3) and the reverse primer NC2 (5-TTAGTTTCTTCCTCCGCT-3) (Zhu *et al*., 2000), and 1.0 U of BIOTAQ DNA Polymerase (Bioline) in a final volume of 50 µl. PCR were carried out using the following parameters: 10 min at 95°C, thirty cycles of 30 s at 95°C, 40 s at 52°C and 75 s at 72°C, with a final extension of 7 min at 72°C. A negative control was included in each amplification. Aliquots of individual PCR products were separated by electrophoresis using agarose gels (1 – 1.5 %), stained with GelRed (25 µg/ml) and detected by the use of ultraviolet transillumination. Gel images were captured electronically and analysed using Bio-Rad's Image Lab software.

The endonucleases *Hinf*I and *Hha*I were used to digest positive amplicons in order to identify larval nematodes at species level. Digestions were performed with incubations of four hours at 37°C. Amplification fragments obtained were separated by 2 % agarose gel electrophoresis and their sizes were determined by comparison with a 100 bp DNA ladder marker (Promega, Madison, WI).

Table 1. Epizootiological parameters obtained for chub mackerels infected by *Anisakis* spp. and *Hysterothylacium* spp. analysed in the present study (CI: confidence interval; NIF: number of infected fish; P %: prevalence indicated as percentage; ml: mean intensity; mA: mean abundance; K: aggregation index).

	NIF	P %	ml	mA	K
***Anisakis* spp.**	151	41.9	5.76	2.42	0.2
(CI)		(36.8 – 47.2)	(4.32 – 8.55)	(1.78 – 3.68)	
***Hysterothylacium* spp.**	158	43.9	2.25	0.98	0.53
(CI)		(38.7 – 49.2)	(1.96 – 2.66)	(0.82 – 1.2)	

Results

On a total of 360 fish examined, a number of 1317 larvae were recovered and morphologically identified as belonging to two genera: *Anisakis* (n= 969) and *Hysterothylacium* (n=348). Specimens belonging to *Anisakis* genus showed morphological features of type I larvae.

The subsample of nematodes analysed using diagnostic molecular keys (n=100) gave positive amplification for 35 specimens, probably due to spoilage of material during the transfer to Rome. All the 35 larvae were found in the viscera. Restriction fragments analyses revealed three distinct banding patterns corresponding to three nematode taxa: *Anisakis pegreffii* (n=16), the hybrid genotype of *A. pegreffii* and its sibling species *Anisakis simplex* sensu stricto (n=1), and *Hysterothylacium aduncum* (n=18). Among the taxa recovered, at least two are considered pathogenic for humans, namely *A. pegreffii* and the hybrid genotype. Two larvae of *A. pegreffii* and the hybrid were found in the same fish specimen, while all other *Anisakis* were from different fish specimens. Regarding *Hysterothylacium*, two larvae were from the same fish specimen and the remaining from different fish specimens.

Regarding the parameters of infection (Table 1), most of larvae were recovered in the visceral cavity, with the exception of nine *Anisakis* spp. larvae recovered in the muscular tissue of three individual hosts (prevalence 1.1 %, CI 0.3 – 2.8). Moreover, the distribution of nematode larvae in the host population resulted strongly aggregated in *Anisakis* (k=0.20) or moderately aggregated in *Hysterothylacium* (k=0.53). Considering prevalence estimations according to the fishing period (Table 2), the highest values of prevalence obtained for *Hysterothylacium* infection was during spring, while *Anisakis* maximum infection level occurred in summer months. The overall differences between infected and non-infected hosts according to seasons were all highly significant for both parasitic species (p≤ 0.00001). When seasonal differences in infection level are evaluated as pairwise comparisons, all combinations showed very significant statistical *p*-values (ranging from 0.0000001 to 0.00027) with the exception of the differences for *Hysterothylacium* in autumn vs. winter (p = 0.0985), and in summer vs. spring (p = 0.0985)

Discussion

The recovery of two pathogenic *Anisakis* species, namely *A. pegreffii* and the *A. pegreffii/A. simplex* s.s. hybrids, in fish intended for human consumption, represents a public health hazard and an economic issue. Previous surveys on the presence of anisakids in fishes collected from the Mediterranean basin have reported high values of infection by *A. pegreffii* in Atlantic chub mackerels from Spain and Sardinia (Casti *et al*., 2017; Madrid *et al*., 2016; Piras *et al*., 2014). Other investigations carried out on *Scomber japonicus* and on *Scomber scombrus* reported the occurrence of *A. simplex* sensu stricto and hybrids in the Western Mediterranean waters (Farjallah *et al*., 2008; Ferrantelli *et al*., 2014). *A. pegreffii* and hybrid forms were also recovered in horse mackerels (*Trachurus trachurus*) caught from two localities off Libyan coasts (Eissa *et al*., 2018). Additional *Anisakis* species, i.e. *Anisakis physeteris* (Goffredo *et al*., 2019) and *Anisakis typica* (Farjallah *et al*., 2008), were observed in the Easternmost Mediterranean waters.

The differential occurrence of anisakid species in distinct areas

Table 2. Prevalence parameter estimated according to seasons (autumn: September, October, November; winter: December, January, February; spring: March, April, May; summer: June, July, August) obtained for Atlantic chub mackerels infected by *Anisakis* spp. and *Hysterothylacium* spp. analysed in the present study (CI: confidence interval; P%: prevalence indicated as percentage; *p*: p value).

	***Anisakis* spp.**		***Hysterothylacium* spp.**	
	P%	**CI**	**P%**	**CI**
Autumn	37.80	27.8 – 48.6	24.40	16.0 – 34.6
Winter	17.80	10.5 – 27.3	33.30	23.7 – 44.1
Spring	50.00	39.3 – 60.7	63.30	52.5 – 73.2
Summer	62.20	51.4 – 72.2	54.40	43.6 – 65.0
p	< 0.00001		< 0.00001	

of the Mediterranean basin may be helpful for the successful use of *Anisakis* larvae for tagging *Scomber* spp. fish stocks, for fisheries management purposes and for the evaluation of food safety in relation to consumers' habits, which could benefit also from the information on the maximum and minimum levels of *Anisakis* infection. Although molecular identification on a larger number of larvae appears needed, the lack of *A.simplex* s.s. records in the area may suggest that the mackerels sampled could belong to a local Mediterranean stock. Conversely, previous findings of this anisakid species in *Scomber scombrus* from Tunisian coasts may be related to mackerel stocks migrating from the Atlantic Ocean through the Gibraltar strait (Farjallah *et al*., 2008).

Parameters of parasitic infection here observed are in agreement with those reported in previous studies carried out in members of the genus *Scomber* (Abattouy *et al*., 2011; Levsen *et al*., 2018). Accordingly, *A. pegreffii* is the prevalent anisakid species reported in mackerels (Abattouy *et al*., 2011; Casti *et al*., 2017; Goffredo *et al*., 2019; Mladineo & Poljak 2014; Piras *et al*., 2014), with the majority of larvae commonly recovered in the visceral cavity. The low number of larvae found in muscles in the present study may represent a feature of potential interest for public health issues, as fillets are the edible part of the fish, thus suggesting a reduced probability of human infection. Mackerels are commonly consumed cooked in Libya, with the exception of the most common traditional Libyan dish based on salted-dry fillets mackerels. Moreover, evidences related to potential human sensitizations for the presence of *Anisakis* antigens also in cooked or salted fish are completely missing for the area under study.

The distribution of nematode larvae in the host population resulted strongly aggregated in *Anisakis* spp. or moderately aggregated in *Hysterothylacium* spp. This is highly common in parasite ecology and may be of interest for control organisms given the potential implication in setting sampling size assessment for routine monitoring and control programs (Shvydka *et al*., 2018).

Regarding the relationships between infection level and seasonal trend observed in *Anisakis*, prevalence values were found higher in spring and summer months rather than in autumn and winter. These results are consistent with those of Akmirza (2003), who reported a higher prevalence of infection by *A. simplex* s.l. in mackerels during spring from two areas of Turkey, and with those of Gutiérrez-Galindo *et al*. (2010) which observed a higher prevalence in summer from Tarragona waters (NE Spain). This information may help both consumers, for the choice of fish species to consume, and policy makers and producers' categories to plan a seasonal fishing strategy aimed at mitigate the health risks related to *Anisakis*, as recently suggested also by Cammilleri *et al*. (2019).

A similar study carried out on *Merluccius merluccius* collected from Libya and on the zoonotic potential in the area advocated that the lack of reported cases of human infection was most likely related to the infrequent habits of consuming raw fish. However, the existence of human infections cannot be excluded given the paucity of awareness for anisakiasis among Libyan physicians (Sharif & Negm-Eldin, 2013).

Conclusions

The continuous monitoring of fish and fish products intended for human consumption is highly recommended by the European Food Safety Authority, with particular attention to *Anisakis* spp., being the only fish-borne parasite able to trigger an allergic response in humans (EFSA 2010). In the present study, Atlantic chub mackerels from south Mediterranean basin resulted infected by three parasitic nematode taxa, and at least two species recovered are considered pathogenic for humans, namely *A. pegreffii* and the hybrid genotype. Levels of infection suggest a residual, but still existing, zoonotic risk for consumers, as a small amount of larvae belonging to pathogenic species was recovered from fish fillets.

Conflict of interest

Authors state no conflict of interest

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