IJP: Parasites and Wildlife 9 (2019) 74–79

Contents lists available at ScienceDirect

IJP: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Air sac trematodes: *Morishitium polonicum* as a newly identified cause of death in the common blackbird (*Turdus merula*)

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ARTICLE INFO

Keywords:
Air sac trematodes
Cyclocoelidae
Death
Histopathological lesions
Morishitium polonicum
Turdus merula

ABSTRACT

Necropsy of two free-ranging common blackbirds (*Turdus merula*) found dead in central Italy revealed the presence of a high number of cyclocoelid flukes in the coelomatic cavity. Cyclocoelid flukes primarily infect avian respiratory system. Histologically, air sac walls were covered with a fibrinous exudate containing degenerate heterophils, many trematodes and some colonies of Gram-positive cocci. The superficial bronchi and parabronchi were markedly distended, and the adjacent pulmonary parenchyma was congested and collapsed. Trematodes, surrounded by a mild suppurative to pyogranulomatous inflammatory reaction, were also observed on the pericardial, intestinal, kidney and hepatic serosal surfaces. The death of the two examined birds was likely due to the high parasite load and associated severe lesions. At parasitological examination, flukes showed a tongue-shaped elongate body, tapered anteriorly and rounded posteriorly, of 2,088–2,314 μm in width and 8,268–11,830 μm in length. The mouth was slightly oval and sub-terminal, with a small oral sucker. The oval pharynx measured 250–309 μm, and the two caeca joined posteriorly. Two large (550–702 μm × 450–520 μm) globular testes were situated obliquely to each other, whereas an oval (250 × 300 μm in mean) or round (about 334 μm in diameter) intertesticular ovary was placed in a longitudinal straight line with the testes. The ootype was about 110 μm in diameter, while the brown-yellow eggs measured 131.5 × 73.9 μm in mean. The genital pore was post-pharyngeal, while the symmetrically arranged vitelline glands were not confluent posteriorly. Morphoflogical diagnosis led to the identification of *Morishitium polonicum*, a cyclocoelid fluke species that typically inhabits the air sacs of blackbirds. The morphological diagnosis was corroborated by molecular phylogenetic analysis of the mitochondrial (CO1, ND1) DNA loci. The present study provides the first report of pathological lesions and death caused by *M. polonicum* in birds.

1. Introduction

The common blackbird (*Turdus merula* Linnaeus, 1758) is a passerine species of the Turdidae family that is found throughout most of Eurasia and North Africa and has been introduced to Australia and New Zealand (BirdLife International, 2016). Sedentary and migratory populations of this bird species may be present at the same latitude (Sitko and Zaleśny, 2014). *Turdus merula* is omnivorous, eating a wide range of insects, earthworms, little snails, seeds and berries. It also occasionally captures small amphibians, lizards and freshwater fishes (Clement et al., 2000). Animal prey predominates, and is particularly important during the breeding season, with berries and seeds taken more in the autumn and winter (Clement et al., 2000). Among bird pathogens, parasites are known to be able to affect negatively the population dynamics of their avian hosts possibly causing retarded growth, weight loss, reduced food consumption, impaired fertility and nesting success, and even mortality, especially among nestlings or when in heavy burdens. Parasites may also cause cyclic fluctuations of wild populations and population crashes and may represent a critical issue in the conservation of threatened species (Hamilton and Zuk, 1982; Snow, 1988; Møller et al., 1990; Loye and Zuk, 1991; Hudson et al., 1998; Thompson et al., 2010; Brym et al., 2018). Although helminth infections are usually not associated with any major health effects, massive infections may in fact result in severe adverse health effects, and death may also occur (Grünberg and Kutzer, 1964; Höfle et al., 2003; Liptovszky et al., 2012).

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https://doi.org/10.1016/j.ijppaw.2019.03.021
Received 5 March 2019; Received in revised form 26 March 2019; Accepted 27 March 2019
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Among helminths of *Turdus* spp., cestode, nematode, trematode and acanthocephalan species have been identified in various organs and systems (Slater, 1967; Martínez et al., 1977; Machalska, 1980; Ching, 1993; Perrucci et al., 1997; Mani et al., 1998; Literák and Sitko, 2006; Okulewicz et al., 2010; Hamer and Muzzall, 2013; Calegaro-Marques and Amato, 2014; Sitko and Zaleśny, 2014). In the respiratory system, the air sac cyclocoelid trematode species *Cyclocoelum mutabile* (Zeder, 1800), *Morishitium dollfusi* (Timon-David, 1950) and *Morishitium polonicum* (Machalska, 1980) have been reported (Martínez et al., 1977; Machalska, 1980; Sitko et al., 2017).

The morphological and molecular identification of a trematode species found in the coelomatic cavity of two deceased *T. merula* and the evaluation of associated pathological lesions, represented the main aims of the present study.

2. Materials and methods

Necropsy of two free-ranging females of *T. merula*, found dead in Jesi (Ancona, central Italy, 43°31′46″ N, 13°14′27″ E) in January 2016 and in Matelica (Macerata, central Italy, 43°15′18″ N, 13°00′41″ E) in March 2017, revealed the presence of a large number of trematodes in the coelomatic cavity (Fig. 1. A, B). Both the two birds were necropsied in a short time after their death. For the identification of flukes at the species level, approximately 50 adult parasites per bird were collected. Most of them were fixed in the medium of Looss (composed of 10% glycerol and 90% of 70% ethanol) and used for parasitological analysis, while the remaining were fixed in 96% ethanol and used for molecular analysis. Pathological lesions associated with fluke infections were evaluated by histopathological analysis.

Adult voucher specimens have been deposited in the collection of Comenius Museum, Přerov, Czech Republic (marked as P-P-1873/5), while DNA samples have been deposited at the Charles University, Third Faculty of Medicine, Prague, Czech Republic (marked as 3LF-4201 and 3LF-4202). Histological slides have been deposited in the archive of the Laboratory of Veterinary Histoptahology, School of Biosciences and Veterinary Medicine, University of Camerino, Matelica, Italy (marked as B16–126 and B17-340).

For parasitological analysis, fixed trematodes were cleared in Amann lactophenol, mounted and microscopically examined. Measurements were taken by means of an ocular micrometer. The identification of the trematode species was performed based on the description given by Machalska (1980).

For molecular analysis, DNA was extracted and amplified as described by Sitko et al. (2017). The primers used targeted the mitochondrial CO1 and ND1 loci (Bowles et al., 1992; Morgan and Blair, 1998; Bray et al., 1999) and the amplified DNA was subjected to bi-directional Sanger sequencing using an ABI 3130 DNA Analyser (Applied Biosystems, Foster City, CA). The resulting consensus DNA sequences were submitted to NCBI GenBank under accession numbers MH800193–MH800195. The newly generated sequences, sequences obtained from NCBI GenBank as of October 31, 2018, and sequences of corresponding outgroups were aligned using MUSCLE (gap opening penalty −400, gap extension penalty 0, clustering method UPGMB, lambda 24) in MEGA5. The alignments were manually corrected for any inconsistencies and the aligned sequences were trimmed to ensure that they all represent the same extent of the analyzed locus. Short-length sequences were removed from the alignments, and only trimmed sequences were utilized for further analyses. The trimmed CO1 locus (partial CO1 coding sequence) corresponded to nt. 49–351 (303 bp) of *Philophthalmus gralli* Mathis and Leger, 1910 JQ675731 (Fig. S1). The trimmed ND1 locus (partial ND1 coding sequence) corresponded to nt. 1–435 (435 bp) of *Parafasciolopsis fasciolaemorpha* Ejsmont, 1932 EF612500 (Fig. S2). For each analysis, the maximum likelihood fits of the 24 nucleotide substitution models (Tables S1 and S2) was calculated. A bootstrap procedure at 1000 replicates and the nearest-neighbor-interchange were used as the maximum likelihood heuristic method of choice to determine the tree inference when the initial tree was formed using a neighbor joining algorithm. Best-fit models for the follow-up maximum likelihood phylogenetic analyses and for the calculations of the evolutionary divergence between the analyzed isolates (Tables S3 and S4) were used. For the CO1 locus, the best-fit model included the assumption of evolutionarily invariable rates, which is not allowed for the calculation of evolutionary divergence. Thus, the best-fit model with the second lowest Bayesian information score was used. The same issue was with the ND1 locus as the divergence analysis is not implemented in MEGA5 for the Hasegawa-Kishino-Yano model.

For histopathology, all organs from the coelomatic cavity were collected and fixed in 10% neutral buffered formalin for a period of 24 h, and routinely processed. Two-µm paraffin sections were placed on Superfrost Plus slides (Histoline, Milan, Italy). The slides were then

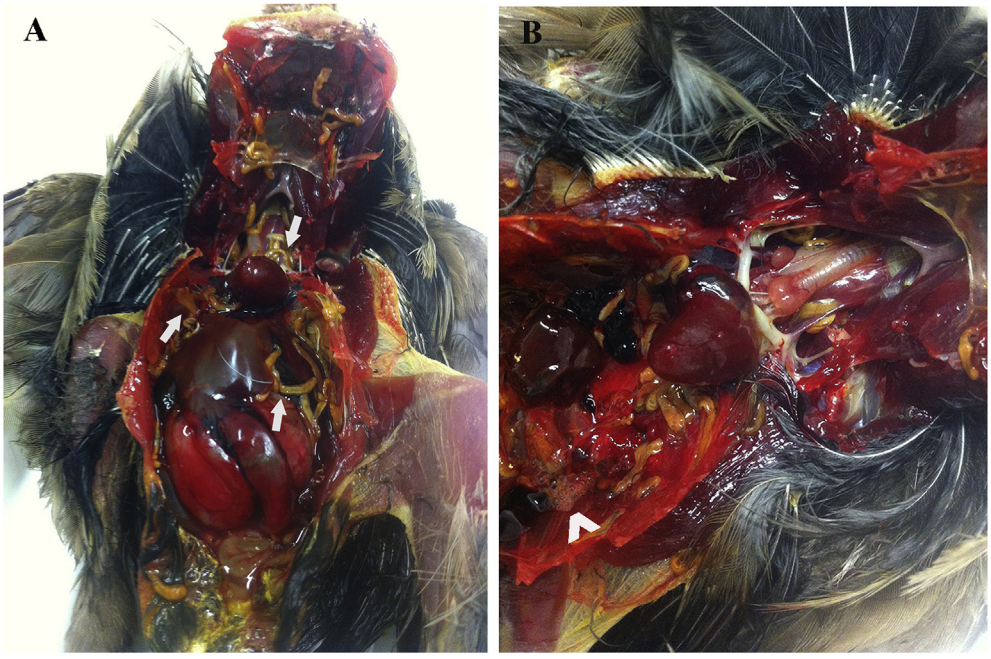

Fig. 1. Necropsy of *Turdus merula*, female. Gross lesions are represented by heavy parasite colonization of coelomic cavity. **A.** Many trematodes are clearly seen on different serosal membranes. Note the presence of parasites on the liver serosa, air sacs and pericardium (arrow). **B.** After removal of all the organs of the gastroenteric apparatus, an involvement of kidney and lungs serosa is also evident. Note the presence of an inflammatory focus with exudate at the periphery of the left lung (arrowhead).

dewaxed and stained with hematoxylin and eosin stain (H&E) for microscopic examination.

3. Results

At necropsy, both birds were found heavily infected by trematodes. More specifically, about 270 adult trematodes were counted in the coelomatic cavity the blackbird from Jesi (Ancona, Italy). Six trematodes were also observed in the choanae. From the coelomatic cavity of the second blackbird (Matelica, Macerata, Italy), 97 parasites were isolated. In both bird subjects, gross lesions were characterized by severe parasite colonization of air sacs, lungs and serosae and were associated with opacification and thickening of the air sacs, where the presence of fibrinous material was visible (Fig. 1. A). After removal of affected air sacs, the lung parenchyma was found congested and showed areas characterized by the absence of gas (atelectasia) for the presence of inflammatory exudate filling the lung parenchyma (Fig. 1B).

Histologically, air sac walls were covered with a mild fibrinous exudate containing degenerate heterophiles, mononuclear cells, cellular debris, and interspersed bacterial colonies of Gram-positive cocci. In many lesions, sections of trematodes were clearly observed inside the exudate. Diffuse areas of calcification with urate precipitates were also present. The superficial bronchi and parabronchi were markedly distended with mucoid material containing bacterial colonies, and the adjacent pulmonary parenchyma was congested and collapsed. Large numbers of trematodes, surrounded by a mild to moderate suppurative to pyogranulomatous inflammatory reaction, were observed also on the pericardial, intestinal, kidney and hepatic serosal surfaces. (Fig. 2).

At parasitological examination, flukes showed a tongue-shaped elongate body of 2,088–2,314 μm in with and 8,268–11,830 μm in length, tapered anteriorly and rounded at the posterior end (Fig. 3A). The mouth was slightly oval and sub-terminal with a weakly developed oral sucker. The ootype was about 110 μm in diameter, while the brown-yellow eggs (Fig. 3B) measured 131.5 × 73.9 μm in mean (range 122–135 μm in length and 62.4–80.6 μm in width). Two large (550–702 μm × 450–520 μm) and globular testes were situated obliquely to each other, while the intertesticular oval (250 × 300 μm) or round (about 334 μm) ovary was placed in a longitudinal straight line with the testes (Fig. 3C). The two caeca joined posteriorly, while the vitelline glands were arranged symmetrically and were not confluent posteriorly (Fig. 3C). The oval and well-developed pharynx measured 250–309 μm, while the genital pore was post-pharyngeal (Fig. 3D). For morphology and dimensions, the fluke here examined was identified as *M. polonicum* according to Machalska (1980), who described this species in *T. merula*. The diagnosis was also consistent with other previously published data of *M. polonicum* in turdids (Visconti et al., 1988; Sitko et al., 2017; Jaume-Ramis and Pinya, 2018).

Molecular analyses of mitochondrial (CO1 and ND1) DNA loci confirmed the morphological diagnosis as revealed by the maximum likelihood approach (Fig. 4). The sequence of the CO1 locus was identical with one of previously published *M. polonicum* sequences (KU877887) and differed by only 0.003 base substitutions per site from other two *M. polonicum* sequences (Table S3). The newly obtained sequences of the ND1 locus of both examined infection cases were identical. The ND1 sequences differed by 0.005–0.012 base substitutions per site from previously identified *M. polonicum* cases from *T. merula* and *Turdus philomelos*, while the closest another previously sequenced cyclocoelid, *Cyclocoelum mutabile*, differed by 0.370–0.386 base substitutions per site (Table S4). Combined, both analyzed markers unanimously confirm the diagnosis of the examined isolates as *M. polonicum*.

4. Discussion

In Europe, *M. polonicum* infections in Turdidae birds have been previously reported in Poland (Machalska, 1980; Sulgostowska and

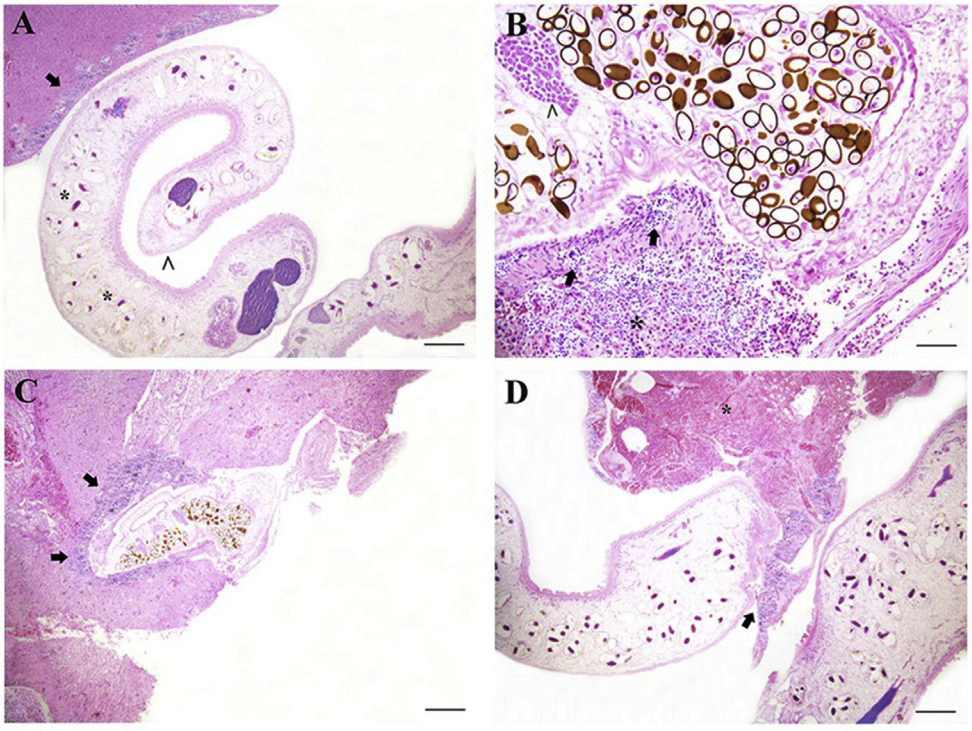

Fig. 2. *Turdus merula* female, histology of different coelomic organs. **A.** Lower power magnification of an area where flukes are adherent to the liver. The presence of inflammatory infiltrate is observed on the Glissonian capsule (arrow) in the contact area with the parasite. At this magnification a general overview of the parasite is also clear: the cephalic portion (arrow-head), different internal organs and the uterus filled by the ova (asterisk) are appreciated (H&E, scale bar = 0.2 cm). **B.** Detail of the contact area between the fluke tegument and liver serosa, involved in the inflammatory reaction (arrow). Note the pyogranulomatous exudate represented by large amounts of mononuclear cells with interspersed heterophils in the area of close contact with the parasite (asterisk). Trematode eggs and an internal gland (arrow-head) are also visible (H&E, scale bar = 200 μm). **C.** Fluke localization on the kidney capsule: note the same inflammatory reaction (arrow) described in the liver, in the areas of more close contact (H&E, scale bar = 2 mm). **D.** Air sacs inflammation and modification in the site of parasite attachment (arrow). Note the previously described inflammatory infiltrate and the altered lung parenchyma (asterisk) in the area of the affected air sac. The pulmonary parenchyma is congested and collapsed, as observed also at gross examination (H&E, scale bar = 200 μm).

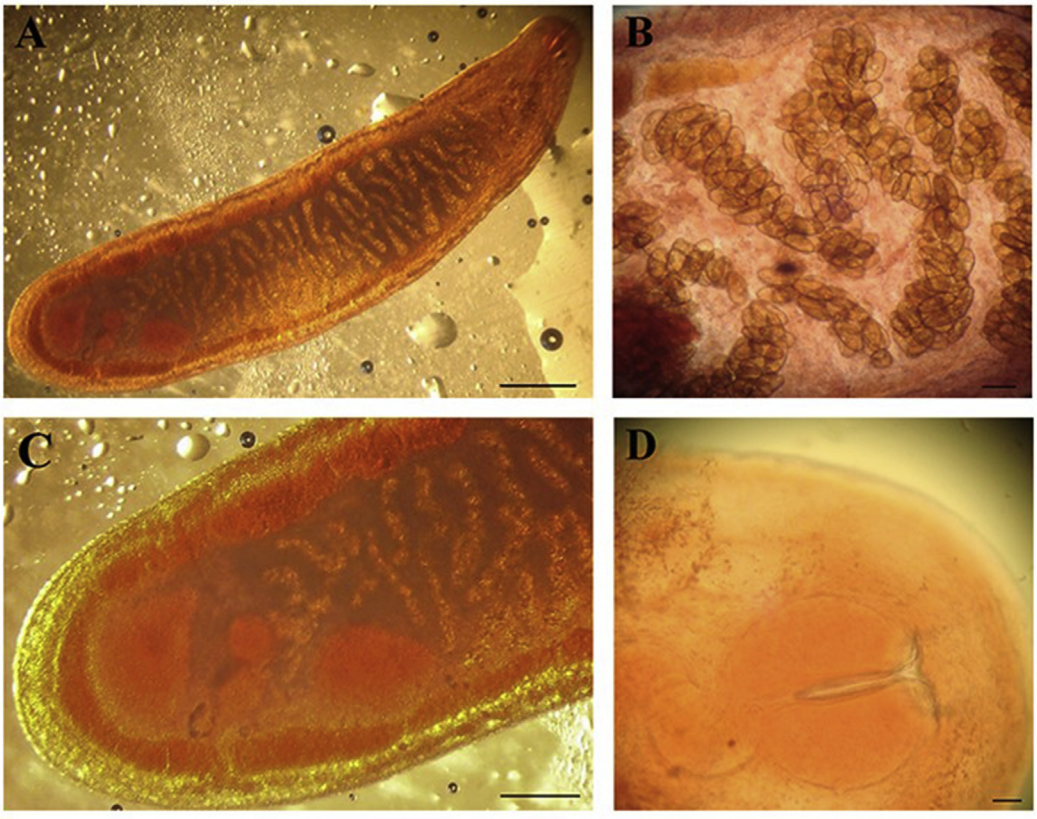

Fig. 3. *Morishitium polonicum* from the air sacs of *Turdus merula*. **A.** Tongue-shaped specimen of *M. polonicum*. In the posterior part of the body are clearly visible the two large testes and the ovary lying between them. (scale bar = 200 μm). **B**. Anterior end of *M. polonicum* showing the eggs inside the uterus. (scale bar = 100 μm). **C.** Posterior end of *M. polonicum* showing two globular testes situated obliquely to each other, an intertesticular oval ovary placed in a longitudinal straight line with the testes, two caeca joined posteriorly and two symmetrical vitelline glands not confluent posteriorly. (scale bar = 300 μm). **D.** The oral sucker, the pharynx, the genital pore of *M. polonicum* in the anterior end (scale bar = 50 μm).

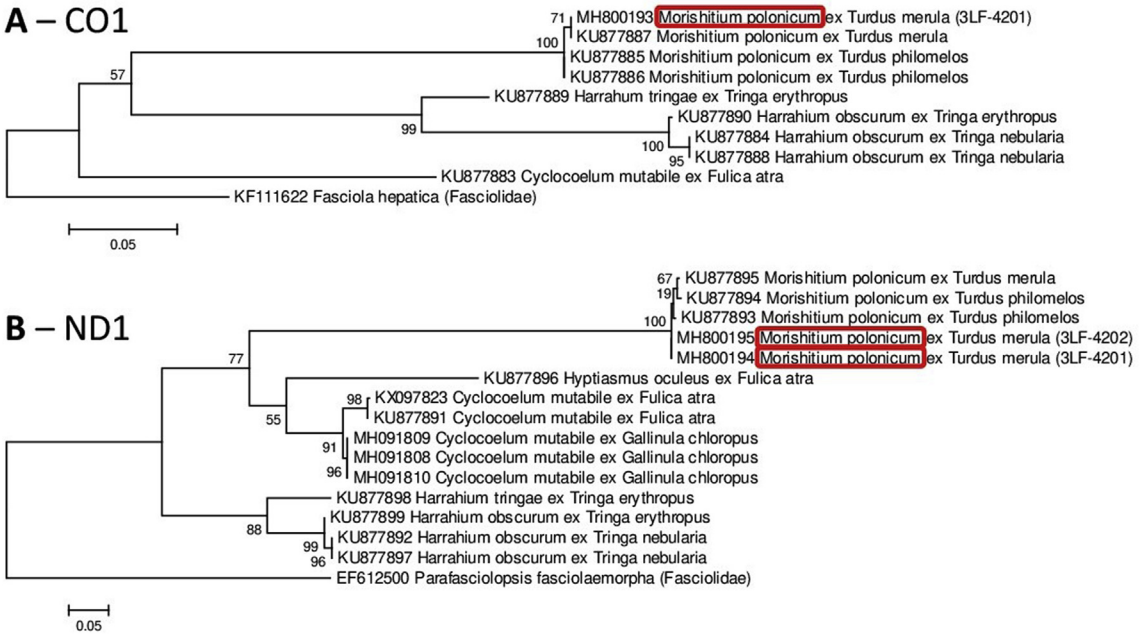

Fig. 4. Maximum likelihood analyses of sequences of mitochondrial DNA loci of the newly isolated *Morishitium polonicum* and previously sequenced Cyclocoelidae. (**A**) CO1, (**B**) ND1. The bars indicate the number of substitutions per nucleotide.

Czaplinska, 1987), Italy (Visconti et al., 1988), Czech Republic (Sitko et al., 2017) and Spain (Jaume-Ramis and Pinya, 2018). In Italy, *M. polonicum* was previously reported in *T. merula* from an area neighboring that where the blackbirds here examined were found (Visconti et al., 1988). Dimensions and morphology of the species reported by Visconti et al. (1988) are very similar to that of the specimens here examined.

Previously, Dronen (2007) and Dronen and Blend (2015) suggested that in the original description by Machalska (1980), the eggs of *M. polonicum* from *T. merula* (150 × 81 μm) are larger compared to those from *Turdus philomelos* (maximum egg size 139 × 69 μm), which was indicative that they are two different *Morishitium* species. Currently, there are recognized four *Morishitium* spp. Infecting thrushes, namely *Morishitium bivesiculatum* (Prudhoe, 1944), *Morishitium dollfusi* (Timon-David, 1950), *Morishitium petrowi* (Oganesov, 1959) and *M. polonicum*. The available material does not allow to test whether all of them represent valid species, but based on morphology and measurements, the species occurring in the two birds here examined matches clearly the measurements provided by Machalska (1980) for *M. polonicum*.

M. polonicum typically inhabits the air sacs of thrushes and its life cycle include terrestrial snails, mainly of the genus *Helicella*, as intermediate hosts (Machalska, 1980; Jaume-Ramis and Pinya, 2018). Adult trematodes reside in the respiratory system and release eggs into the air sacs of the definitive avian host. These eggs are then excreted via respiratory secretions. In the environment, eggs hatch into miracidia, which asexually reproduce within the snail intermediate host, after burrowing into its muscular foot. Miracidia then develop into rediae to produce the metacercariae, the infective life stage. The definitive avian host consumes the intermediate host containing the metacercaria, and adult fluke development proceeds (Delaski et al., 2015).

In wild animals, parasites may negatively affect the health, survival, growth and reproduction (Norte et al., 2009; Thompson et al., 2010, 2013; Brym et al., 2018). Moreover, in wild animals a huge diversity of factors, as seasonal migration, environmental changes, human interventions in wild areas, immunology and diets, including changes in food and habits, may influence or increase the risk and susceptibility to parasitic infections at the individual or population level (Thompson et al., 2010, 2013; McDonald et al., 2018; Moudgil and Singla, 2013; Hawley and Altizer, 2011; Van Hemert et al., 2014). Many parasitic helminth species often responsible for significant disease in their animal hosts, are transmitted by the ingestion of intermediate hosts. Therefore, host diet and food habit may play an important role in the acquisition of these infections (Leung and Koprivnikar, 2016). Moreover, experimental studies have indicated that effects of parasitism can vary with host sex (Granroth-Wilding et al., 2017). Interestingly, it was previously observed that female birds of the family Turdidae are more frequently infected with *Moristhitium* spp. than males (Okulewicz and Sitko, 2012). To explain this finding, authors suppose that this may be linked with differences in feeding preferences between male and female turdids, since females may prefer eating snails for the intake of calcium necessary to build eggshells and ingested snails may contain the infective stages of *Moristhitium* trematodes. Nevertheless, this same reason may also represent the main factor for the high parasite load and the consequent death observed in the two female blackbirds examined in this study.

Although suspected (Visconti et al., 1988; Okulewicz and Sitko, 2012), no previous reports deal with disease and death caused by *Morishitium* spp. trematodes. Nevertheless, severe clinical signs and lesions, debilitation and death associated with infections caused by different air sac cyclocoelid trematodes, have been reported in several avian species, including free-ranging and captive animals (Cole et al., 1995; Delaski et al., 2015). In free-ranging birds, a female snail kite (*Rostrhamus sociabilis plumbeus* Ridgway, 1874 (Accipitriformes) from Florida (USA) found in poor condition and deceased during the transport to a raptor rehabilitation unit, was diagnosed with a heavy infection caused by the cyclocoelid air sac trematode species *Bothrigaster variolaris* Fuhrmann, 1904, which was considered the main contributing factors for the death of the bird (Cole et al., 1995). Necropsy of an American coot (*Fulica americana*) found dead in USA, revealed an infection caused by the air sac trematode *C. mutabile* associated with biliary congestion, hemopericardium, blood-filled air sacs, ruptured aorta and secondary bacterial infection (Branton et al., 1985). *C. mutabile* may also infect thrushes, and a prevalence of about 8% and an average intensity of about 17 parasites per bird were recorded in 90 examined *Turdus* spp. birds (Cardells et al., 2014). In avian exhibits of zoos and captive enclosures, air sac trematode infections have been frequently accounted as a cause of retardation of molting, reduced breeding performance, debilitation and death in several bird species (Libert et al., 2012; Delaski et al., 2015). Stressing factors linked with captivity are likely to exacerbate the consequences of these infections. Death often occurred after birds showing dyspnea due to physical bronchial obstruction and suffocation (Libert et al., 2012; Delaski et al., 2015). Several difficulties in the treatment and control of air sac trematode infections have been also reported (Dronen et al., 2009; Libert et al., 2012; Delaski et al., 2015). The life cycles of these trematodes, that include snail intermediate hosts and frequently including more than a single bird species as vertebrate definitive hosts in the life cycle of a single cyclocoelid trematode species, are considered important factors that in these facilities make the management and control of air sac trematode infections very difficult and a potentially significant problem (Dronen et al., 2006). Passeriformes are considered at greater risk of air sac trematode infections and lesions caused by these parasites than other birds (Dronen et al., 2006, 2009). Among captive Passeriformes, death associated with air sac trematodiasis caused by *Szidatitrema* species, have been reported in *Irena puella* (Latham, 1790) and *Thraupis episcopus* (Linnaeus, 1766) (Delaski et al., 2015). In other captive avian species, severe clinical signs and death have been reported in *Lybius dubius* (Gmelin, 1788) heavily infected with *Szidatitrema yamagutii* Dronen et al. (2006) and, mainly, in *Momotus momota* (Linnaeus, 1766) infected with *Circumvitellatrema momota* Dronen et al. (2009) (Dronen et al., 2009; Libert et al., 2012; Delaski et al., 2015).

Although infections caused by *M. polonicum* were previously reported in blackbirds and in other European turdids (Machalska, 1980; Sulgostowska and Czaplinska, 1987; Visconti et al., 1988; Sitko et al., 2017; Jaume-Ramis and Pinya, 2018), associated pathological lesions were never reported before. However, as observed in the blackbirds examined in this study, severe cyclocoelid air sac trematode infection with associated airsacculitis, often fibrinous, pyogranulomatous or granulomatous, and with pyogranulomatous bronchitis and peribronchitis and secondary bacterial infections, have been reported in different species of birds, including both Passeriformes and other avian taxa, deceased after infections by cyclocoelid air sac trematodes (Cole et al., 1995; Libert et al., 2012; Delaski et al., 2015). Bronchiectasia, atelectasia and pulmonary edema observed in this study in the two examined blackbirds, were previously reported also in a raptor (Cole et al., 1995).

5. Conclusions

Parasitological, molecular and pathological analysis performed in this study identified the air sac trematode species *M. polonicum* as the cause for death of these two common blackbirds (*T. merula*). Until now, the pathogenicity of *M. polonicum* was poorly understood because of the lack of recorded clinical cases and pathological lesions. Despite in some previous observations *Morishitium* spp.-infected and deceased birds were found in poor conditions (Visconti et al., 1988; Okulewicz and Sitko, 2012), these findings did not definitely allow to identify these flukes as the main cause of the observed signs. Nevertheless, the diffuse and highly severe gross and, mainly, histological lesions found associated with *M. polonicum* infection in the two blackbirds here examined, indicate that when in high infection load, this fluke is responsible for severe lesions involving all the organs of the coelomic cavity. As

highlighted in this study, these lesions may also be complicated by other secondary pathogens. Moreover, severe histological lesions here observed in the areas where *M. polonicum* adults were in contact with the serosae of the two examined blackbirds, may represent a further indication of a direct involvement of this parasite in the etiology of observed lesions. The present study is the first report of pathological lesions and death caused by *M. polonicum* in the common blackbird.

Declarations of interest

None.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgements

We thank Miroslav Těšínský for expert technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.03.021.

Financial support

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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