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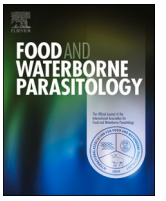

Fasciola hepatica: Updates on egg morphology, host range, and distribution

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ABSTRACT

During a survey for helminths in reindeer (*Rangifer tarandus*) across the Palearctic region, eggs were found in zoo reindeer feces. These were identified as eggs of *Fasciola hepatica* based on their morphology, morphometrics, location, and analysis of their partial sequence of ITS rDNA region. Some of the eggs had an appendage, previously unreported. Additionally, adults of *F. hepatica* were studied. Eggs derived from their uteri were also appendaged. Diagnostic morphological traits of *F. hepatica* eggs (abopercular appendage, knob, egg shell thickening, and egg shape) are discussed in this article. Three dimensional models of *F. hepatica* eggs were created to demonstrate the eggs features as best as possible. Since fecal examination remains gold standard in diagnosing fasciolosis in humans and animals worldwide, our findings may contribute to improved diagnostics. This research has also shown that reindeer can be a final host for *F. hepatica*. We also discuss whether the Novaya Zemlya archipelago might be the northernmost site of fasciolosis.

1. Introduction

Disease caused by *Fasciola hepatica* in humans was estimated as "of most public-health importance" by the World Health Organization in its report on neglected tropical diseases (WHO, 2013, p. 85). Fasciolosis in humans and animals was recognized in more than 50 countries worldwide, affecting all inhabited continents (Esteban et al., 1998; Tanabe et al., 2024). Over 17 million infected people were reported by the end of the XX century (Hopkins, 1992; Mas-Coma et al., 2009; Mas-Coma et al., 2014a). Present day estimations consider 91 to 180 million people (Flores-Velázquez et al., 2023) and over 700 million animals to be at risk of infection annually (Drescher et al., 2023). Not to mention profound health damage due to fasciolosis and all the related dramatic costs for medicine (both human and veterinary) and farming, there was a negative impact even for the environment found: sick ruminants emit more greenhouse gas into the atmosphere (Hayward et al., 2021).

A first step on the way to control fasciolosis is its diagnostics. Different approaches of life-time lab diagnostics were developed,

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including clinical blood test, immunodiagnostics (serological tests), allergy test, molecular techniques, etc. Among them a fecal examination is recognized as the gold standard (Pantelouris, 1965; Mas-Coma et al., 2014b; Drescher et al., 2023). Coprological techniques are numerous and various, but all of them reveal eggs of *F. hepatica* (Mas-Coma et al., 2014b). Therefore morphology of these eggs have been studied in great detail for over a century (Sinitsin, 1930; Køie et al., 1976; Valero et al., 2009).

The findings described here were generated as part of a broader large-scale research, on helminths in reindeer (*Rangifer tarandus*) across the Palearctic region started in 2018. The present article describes cases where eggs resembling those of *Fasciola* were found in two reindeer. Our serendipitous findings may be useful in terms of global coprological diagnostics of fasciolosis in humans and animals, including differential diagnostics. Also this work contributes to the expansion of the list of final hosts for *F. hepatica*. Finally, it offers a hint of the northern extend of *F. hepatica*.

2. Materials and methods

2.1. Background

Our survey for helminths in *R. tarandus* across the Palearctic region involved regular fecal analyses of sample sets obtained from different types of reindeer: wild, semi-wild (farm, sled, experimental), zoo. The first case involved a fecal sample from a reindeer, located in zoo. Numerous eggs, resembling those of *F. hepatica*, were found in its feces. Yet some of their morphologic traits indicated the possibility of *Fascioloides magna*. Efforts in fecal sampling were made to rule out spurious parasite, and to get more material for analysis. While trying to identify these eggs via DNA analysis, a parallel search for the adults of *F. hepatica* was initiated to compare their eggs with those found in feces. Several live liver flukes derived from the bile ducts of a slaughtered bull were provided by a veterinary inspector, and were studied genetically and morphologically. The second case related to wild reindeer from the Novaya Zemlya archipelago (*Russian Arctic* National Park). From a fecal sample set, a single *F. hepatica* egg was found. Details of the samplings are presented in the Table 1 and in the Fig. A1 (Appendix A).

2.2. Fecal sampling

In the first case freshly excreted feces were picked up from the ground. A total of six reindeer were in a pen. Animals were distinguished according to different fur color, age and sex. Each fecal sample was placed into an individual container labeled with reindeer name and collection date. These were stored at +4 °C and were delivered to the lab within two days.

In the second case feces were picked up from the ground shortly after their excretion by a male group of wild reindeer spotted by reserve employees. Novaya Zemlya reindeer (*R. t. pearsoni*) are protected due to their conservation status; therefore, disturbance was avoided. Nine fecal samples were collected and placed into individual zip-lock bags labeled with coordinates and date. These were stored at −18 °C until delivery to the lab 1.5 month later.

2.3. Adults sampling

Adult liver flukes were obtained within a piece of liver at the slaughterhouse. It was placed in a jar filled with saline. As liver flukes were captured alive, they moved and soon left the bile ducts. They were then placed into another container with fresh saline and delivered to the lab within a day. Preliminary identification was based on their typical leaf shape with “shoulders” at the anterior end, along with their localization, host species, and location.

2.4. Fecal examination

Coprological analysis was performed in accordance with the National Standard of the Russian Federation GOST R 54627–2011 *Ruminant animals. Methods of Laboratory Helminthological Diagnostics*, namely Section 9.2 *Sequential washing method for diagnosing fasciolosis, paramphistomatosis, dicrocoeliosis and other helminthiases*. It prescribes: homogenize feces using a mortar and pestle; add water (50 ml for 3 g of feces); mix it carefully; filter the mixture (two layers of synthetic fiber with 1 mm mesh diameter were used); settle the mixture for 5 min until a sediment appears; pour off supernatant; resuspend sediment with the same amount of water; settle the suspension for another 5 min; repeat until the supernatant layer is transparent. The sediment was then poured onto slides and examined under a microscope.

Table 1
Collection data for samples from reindeer and cattle potentially contained *F. hepatica*.

Host species	Material	Location	Coordinates (decimal degrees)	Date collected
Rangifer tarandus (zoo female)	feces	Leningrad Oblast, Russia	59.594384 N, 30.283104 E	24-May-2023 27-Jun-2023 31-Aug-2023
Rangifer tarandus (wild male)	feces	Severny Island, Novaya Zemlya archipelago, Arkhangelsk Oblast, Russia	76.784423 N, 68.823853 E	07-Jul-2023
Bos taurus (beef male)	liver	Tula Oblast, Russia	53.555201 N, 38.190402 E	14-Jul-2023

2.5. Adults examination

Dead adult worms were placed in Petri dishes with tap water and cut with a scalpel around the uterus area. Eggs released into the water were collected using a pipette and placed onto slides for microscopy.

2.6. Eggs imaging

Eggs derived from both feces and adults were placed onto slides in a drop of tap water and covered with cover slips (24 × 24 mm). Light micrographs were taken with 40× and 100× objective lens. Morphometry was based on the obtained micrographs using Fiji/ImageJ Version 1.2.4 RRID:SCR_003070 software (National Institutes of Health, USA) in straight line and freehand line modes. The program used a microscope calibration slide (transmitted light stage micrometer) OMP (LOMO-MA, Russia).

Additionally 3D models of *F. hepatica* eggs were created by an artist (Appendix B). Our own original light microscopy (LM) photographs and scanning electron microscopy (SEM) images available in the scientific literature (Køie et al., 1976; Hussein et al., 2010a, 2010b; Gherbawy et al., 2013; Taha et al., 2014) served as references for those models. Modeling was performed using Blender Version 3.9 RRID:SCR_008606 software (Blender Foundation, Netherlands). Sculpting was performed using ZBrush Version 4R7 software (Maxon, Germany). Then 3D models were rendered via Blender to obtain 2D images resembling SEM ones.

2.7. DNA analysis

In the first case there were enough eggs to extract DNA. The eggs were obtained via the sedimentation coprological technique. They were stored in tap water in watch glasses for two weeks for miracidia to develop. The eggs were collected individually using eyelash handmade manipulator (Halbritter et al., 2018). Three pairs of 1.5 Eppendorf tubes (a pair per sample set) were filled with tap water, and at least 65 eggs containing live miracidia were placed in each one. The tubes were placed in a freezer (−70 °C) to destroy the egg shells, and the QIAamp DNA Accessory Set, Micro kit (Quiagen, Netherlands) used to extract DNA. To identify trematode species internal transcribed spacer (ITS1, 5.8S, ITS2) was amplified using primers BD1, BD2 (Luton et al., 1992) and protocols as described previously (Mahami-Oskouei et al., 2011).

Three adult worms served as separate sources for DNA analyses. DNA was extracted using the QIAamp DNA Accessory Set, Mini kit (Quiagen, Netherlands). The partial sequence of ITS rDNA region was amplified as described above. Additionally small subunit ribosomal ribonucleic acid (SSU rRNA, or 18S) and large subunit (LSU, or 28S) were studied using primer pairs WormA, WormB and ZX-1Fa, 1500R, respectively. Primers and protocols were as described by Littlewood et al. (Littlewood et al., 2008). The results of PCR were visualized in a 1% agarose gel. Then bands of expected molecular weight were excised, and DNA was extracted using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA). The PCR products were cleaned by an ethanol precipitation in the presence of ammonium acetate. Sanger dideoxy sequencing was performed by Genotech® (Moscow, Russia). The obtained chromatograms were analyzed using Chromas 2.6.6. RRID:SCR_000598 (Technelysium Pty Ltd., Australia). Program BLASTN 2.13.0+ was used to search for sequences in GenBank similar to those of the studied material (Zhang et al., 2004).

In the second case there was only one egg of interest without an opportunity of double sampling, therefore no DNA analysis was possible.

3. Results

The measurements of eggs were: 71.2 (±3.75) × 128.9 (±9.08) μm ($n = 30$) in the first case and 77 × 120 μm ($n = 1$) in the second case (Table A1, Appendix A).

The ITS sequences (900 bp) were obtained from eggs derived from reindeer feces, as well as ITS sequences (900 bp), 18S sequences (814 bp), and LSU sequences (718 bp) were obtained from adults derived from bull's liver. As results were the same for every approach, single sequences of each type were uploaded to GenBank. Voucher and GenBank accession numbers of *F. hepatica* recovered from reindeer feces and bull's liver are presented in the Table 2.

Appearance of *F. hepatica* eggs derived from zoo reindeer feces and from an adult fluke is presented in Fig. 1. Pictures were obtained by light microcopy. Appearances of *F. hepatica* eggs created by an artist under scientific supervision is presented in Fig. 2. Pictures were obtained by rendering of 3D models. Light microscopy of overall shape and abopercular pole structure in *F. hepatica* eggs derived from

Table 2
Voucher and GenBank accession numbers of *F. hepatica* recovered from reindeer feces and bull's liver.

Host species	Developmental stage	Voucher[1]	GenBank[2]
Rangifer tarandus	embryonated eggs	IPEE_Parasites 14320	PP328913 (for ITS)
Bos taurus	adult	IPEE_Parasites 14321	PP330400 (for ITS)
Bos taurus	adult	IPEE_Parasites 14321	PP328921 (for 18S)
Bos taurus	adult	IPEE_Parasites 14321	PP328922 (for 28S)

[1] Voucher specimens with definitive identifications and accession numbers archived in the Museum of Helminthological Collections of the Parasitology Center at the A. N. Severtsov Institute of Ecology and Evolution of the Russian Academy of Sciences (Moscow, Russia).
[2] GenBank accession numbers for sequences from a single adult or 65 embryonated eggs of *F. hepatica*.

reindeer feces are presented in Fig. 3. Light microscopy of an egg of *F. hepatica* derived from the wild reindeer feces (from Novaya Zemlya archipelago) is presented in Fig. 4.

4. Discussion

All the sequences obtained in this study were 100.00% consistent with the sequence from adult *F. hepatica* in cattle in Australia deposited by Le et al. under accession number MN970007 (Le et al., 2020). Thus, morphological identification of *F. hepatica* eggs from zoo reindeer feces and adult flukes from bull's liver was supported genetically.

4.1. Diagnostic traits of egg morphology

4.1.1. Abopercular appendage

Two species of liver flukes were suspected to infect zoo reindeer (the first case): common liver fluke (*F. hepatica*) and giant liver fluke (*F. magna*). The former has been reported for reindeer in USSR (Mizkewitsch, 1967), and the latter was reported for caribou in North America (Kutz et al., 2019). Being originally a Nearctic trematode *F. magna* was introduced to Europe in 1875 (Swales, 1935). Since then *F. magna* was reported in different game species (also introduced to Europe) and local ruminants, but not for *R. tarandus* (Králová-Hromadová et al., 2016). Both common and giant liver flukes inhabit liver, but the former prefer bile ducts, whereas the latter is usually encysted in parenchyma. This difference determines the choice of treatment. That is why an accurate diagnostics was needed for the zoo reindeer.

Some of the obtained eggs had appendages on their abopercular poles (Fig. 1A, B, C) as described and drawn by Sinitsin for *F. magna* (Sinitsin, 1930, 1933; Skrjabin, 1948). Drawings by Skrjabin show no appendage in *F. hepatica* eggs (Skrjabin, 1948). Sinitsin (1930) stated that "neither in size nor shape do the eggs of *F. magna* differ from those of *F. hepatica*, but they [*F. magna*] are furnished with an appendage, a sort of filament, by which they can be easily identified". According to Sinitsin, in the uterus of adult almost all *F. magna* eggs are appendaged, and only about 20% of eggs maintain their appendages after excretion with feces.

As a case of co-infection of ruminant with *F. magna* and *F. hepatica* was reported in Europe (Leontovyč et al., 2014), there might have been a mixture of eggs of two species. Thus, adults of *F. hepatica* were needed to study their eggs. Consequently, *F. hepatica* eggs, derived from the uterus, also had appendages (Fig. 1D, E, F).

Sinitsin (1930) studied both liver fluke species, and surprisingly did not notice an appendage in *F. hepatica*. Perhaps, he employed different methods to process the eggs. Thus, the sieves cause the appendages to break off (Swales, 1935). Swales wrote that the site from where an appendage protrudes in *F. magna* egg "is situated slightly to one side of the middle line of the egg; it varies from 4 to 21 μm in length" (Swales, 1935). Our study showed that appendages of *F. hepatica* eggs are situated slightly asymmetrically too, their length from 10 to 18 μm (Fig. 3C). Since these appendages are easily broken, their length may not be a reliable criterion for differential diagnostics. An appendage presence itself, its length and position only increase the similarity between eggs of *F. hepatica* and *F. magna* which is not surprising, for these two species are close relatives.

4.1.2. Abopercular knob

In some cases an abopercular knob can be seen on the egg of *F. hepatica*. It was also referred to as "protuberance" ["бугорок" in Russian] (Skrjabin, 1948) and "bouton" [in French] (Kremer and Chaker, 1983). It is supposedly nothing else but a remnant of a broken appendage.

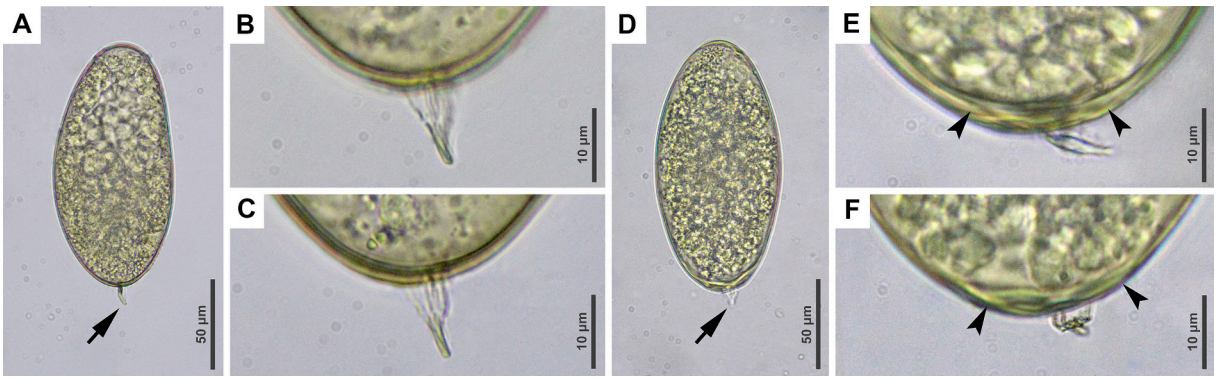

Fig. 1. Light micrograph images of *F. hepatica* eggs. Egg obtained from reindeer feces and captured at 400×, operculum upward (A). Note an appendage on its abopercular pole (arrow). Abopercular pole of the same egg captured at 1000×, focused on the left (B) and on the right (C) side of an appendage. Egg obtained from an adult and captured at 400×, operculum upward (D), an appendage on its abopercular pole (arrow). Abopercular pole of the same egg captured at 1000× (E) and after the egg was slightly rotated around its longitudinal axis of symmetry (F). Note the eggs shell thickening (arrow heads).

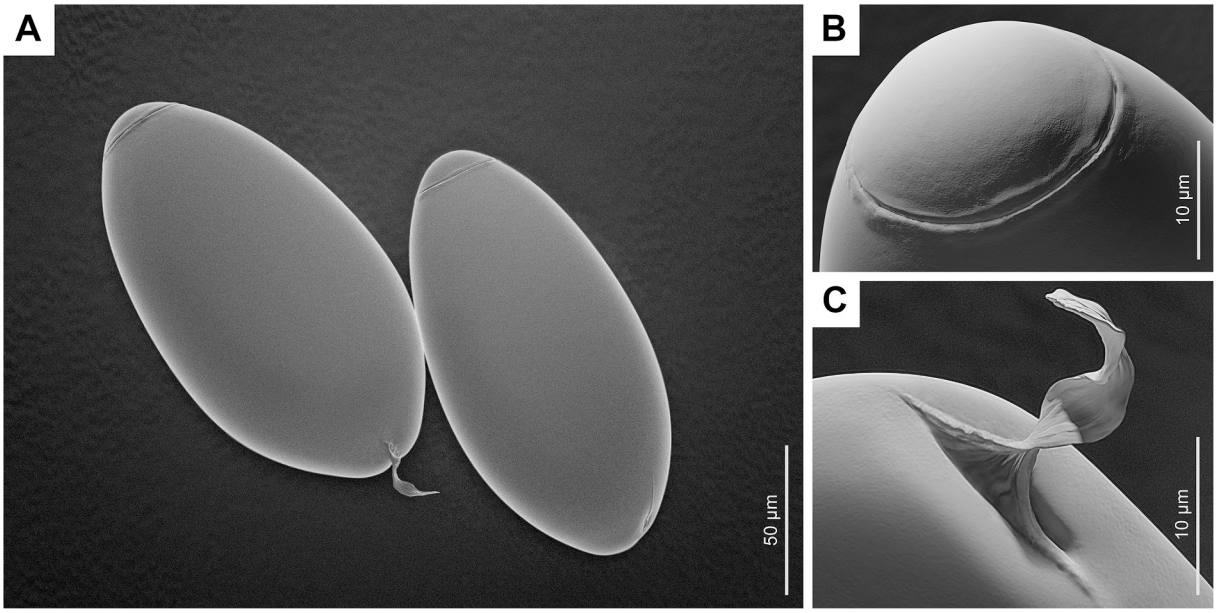

Fig. 2. Appearances of *F. hepatica* eggs (not real SEM images, but renders of 3D models). (A) Appearances of two eggs: the one with an appendage and without a thickening of its abopercular pole (left), and the one without an appendage and with a thickening of its abopercular pole (right). (B) Operculum of an egg. (C) Abopercular pole of an egg with an appendage and shell thickening.

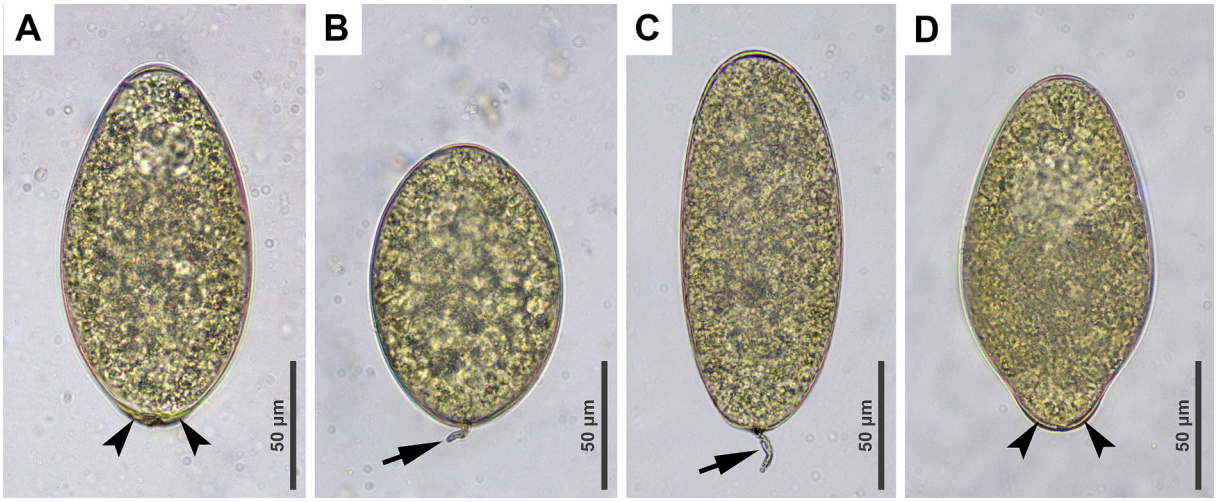

Fig. 3. Light microscopy of overall shape and abopercular pole structure in *F. hepatica* eggs derived from reindeer feces. All eggs are captured at 400×, operculum upward. (A) Ovoid egg shape, shell thickening (arrow heads). (B) Shortened ellipse egg shape, abopercular appendage (arrow). (C) Elongated ellipse egg shape, abopercular appendage (arrow). (D) Compound egg shape resembling an adult *F. hepatica* (oral sucker downward) with an egg shell thickening (arrow heads).

4.1.3. Abopercular egg shell thickening

Swales (1935) described abopercular egg shell thickening as another morphological trait by which he believed eggs of *F. magna* and *F. hepatica* could be easily discriminated. However, our own findings demonstrate presence of the same egg shell thickening in some eggs of *F. hepatica* (Figs. 1D, E, F; 3A, D) reaching to three times the thickness of other parts of the shell. The same has been already demonstrated for *F. hepatica* eggs (Kremer and Chaker, 1983, Fig. 1C). Published LM images of *F. magna* eggs (Kutz et al., 2019, p. 198; Verocai et al., 2020, p. 136; Halász et al., 2023) show no egg shell thickening. Thus, an egg shell thickening is not always present in both *F. hepatica* and *F. magna* and cannot serve as a criterion to distinguish these two species.

Hussein et al. (Hussein et al., 2010a, 2010b) performed LM and SEM studies of eggs of *F. hepatica* and *F. gigantica* paying attention to the abopercular pole. They reported an "umbilicus-like invagination" on the abopercular pole of *Fasciola* eggs and suggested that some debris situated there may resemble a knob (Hussein et al., 2010a, 2010b, Figs. 2 and 3). Indeed, the eggs depicted via SEM are often

Fig. 4. Light microscopy of an egg of *F. hepatica* found in feces of Novaya Zemlya reindeer. Note an operculum (arrow heads) and undivided germ cell (arrow). An egg was captured at 400 × .

contaminated with different debris, and during LM sessions eggs can be seen covered with various debris and microbes. However, what can be seen on the SEM images by Hussein et al. may be interpreted as abopercular poles with egg shell thickening and a fragment of broken appendage situated in an invagination (following their analogy, an appendage can be compared to a piece of umbilical cord). It is the thickening that allows that invagination. Therefore, those "umbilicus-like invaginations" are supposedly also optional. This point of view is supported by Kremer and Chaker, for as yolk cells release less granulations "the shell will have a uniform thickness" (Kremer and Chaker, 1983).

In contrast to LM offering a lateral view on the egg, SEM allows more different angles (points of view). Since the appendages were reported to be highly fragile (Swales, 1935) the present study did not perform an actual SEM, but created SEM-resembling images to demonstrate egg structures as best as possible (Fig. 2).

4.1.4. Egg shape

Variety in egg shape among Fasciolidae was noted long ago (Barlow, 1925; Swales, 1935). Our study has also revealed egg shape diversity (Fig. 3) including ovoid, ellipsoid (shortened, regular and elongated), compound egg shape (resembling an adult *F. hepatica*), and all in between cases. Swales (1935) suggested that such shape variety was due to the locomotion of miracidia within the eggs. In our study an egg shape of *F. hepatica* did not change along with miracidium development and did not depend on its movements. Shape diversity was obvious in unembryonated eggs from the very beginning. Swales called broader eggs "abnormal" (Swales, 1935). Yet, an egg of *F. hepatica* shaped like elongated ellipse was SEM depicted (Gherbawy et al., 2013, Fig. 3) as a control (in contrast to treated eggs). Probably, it is more the variety than abnormality, as the shape did not affect miracidia development.

4.2. Host range

Eggs of *F. hepatica* occur spuriously via predation or coprophagia (Vogel, 1922; Skrjabin, 1948). Reindeer are coprophagous (Turkin, 1900). As humans can be final hosts for *F. hepatica*, and reindeer can eat human feces, the latter can excrete *F. hepatica* eggs without being infected themselves. Thus, feces from zoo reindeer (the first case) were sampled three times to rule out spurious parasite. Each time numerous eggs were genetically confirmed as *F. hepatica*. Thus, a reindeer (*R. tarandus*) should be considered as a final host for *F. hepatica*.

4.3. Distribution

In the second case an egg found in the feces of a wild reindeer (Fig. 4B) from the Severny Island of the Novaya Zemlya archipelago met criteria for both *F. hepatica* and *F. magna* in terms of its shape (ovoid), color (light brown), structure (operculum, germ cell close to it), and size (77 × 120 µm). Given its possible connection to the mainland, it is more likely *Fasciola*, as it was reported from neighboring areas (Mizkewitsch, 1967). If so, it might be a true parasite of that reindeer or a spurious parasite. Because only one sample was available, there was no opportunity to find out if a reindeer was parasitized by liver fluke. The fact, that the egg was single, does not necessarily support the version of a spurious parasite, because there is no direct correlation between number of flukes and number of excreted eggs (Valero et al., 2002). Recent studies indicate significant taxonomic diversity of malacofauna in northern islands of Russia, numbering dozens of species of mollusks (Bespalaya et al., 2022). Thus, *Galba truncatula* (formerly known as *Lymnaea truncatula*), an intermediate host for *F. hepatica*, may be found on the islands of Novaya Zemlya. This also indirectly suggests the possibility of the presence of liver flukes on the island. This case might provide a good direction for further investigation into the northern limits of *F. hepatica*.

5. Conclusion

Eggs of *F. hepatica* may or may not have abopercular appendage, knob, and egg shell thickening. Thus, these structures cannot serve as criteria to distinguish *F. hepatica* and *F. magna*. Eggs of *F. hepatica* may be ovoid, ellipsoid (shortened, regular and elongated), or have a compound shape (resembling an adult *F. hepatica*), or variations of these. Since fecal examination remains the most recommended diagnostic method, specialists worldwide should be informed about the morphological diversity of *F. hepatica* eggs for more reliable veterinary and medical diagnostics. Reindeer (*R. tarandus*) should be included in the list of the final hosts for *F. hepatica*. Novaya Zemlya archipelago might be the northernmost site of fasciolosis, but further investigation is required.

Declaration of generative AI in scientific writing

During the preparation of this work the authors did not use any AI-tool / service.

CRediT authorship contribution statement

Olga Loginova: Writing – review & editing, Writing – original draft, Resources, Investigation, Conceptualization. **Boris Efeykin:** Writing – review & editing, Funding acquisition. **Anna Krutikova:** Writing – review & editing, Resources. **Ivan Mizin:** Writing – review & editing, Resources. **Sergei Spiridonov:** Writing – review & editing, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A

Sampling sites for reindeer feces contained eggs of *F. hepatica* and for adults of *F. hepatica* from the bull are shown in Fig. A1. The measurements of *F. hepatica* eggs recovered from the feces of zoo reindeer (Leningrad Oblast) and wild reindeer (Novaya Zemlya archipelago) are presented in the Table A1.

Fig. A1. Map of the Palearctic region indicating the sampling sites in the Russian Federation: 1, Leningrad Oblast; 2, Tula Oblast; 3, Severny Island of the Novaya Zemlya archipelago, Arkhangelsk Oblast.

Table A1
Measurements of *F. hepatica* eggs recovered from the feces of zoo reindeer (Leningrad Oblast) and wild reindeer (Novaya Zemlya archipelago).

Egg's number	Zoo reindeer (Leningrad Oblast)		Wild reindeer (Novaya Zemlya archipelago)	
	Width	Length	Width	Length
01	72	130	77	120
02	74	140	–	–
03	73	129	–	–
04	73	145	–	–
05	69	136	–	–
06	66	129	–	–
07	76	134	–	–
08	74	141	–	–
09	71	126	–	–
10	73	122	–	–
11	72	141	–	–
12	75	132	–	–
13	72	133	–	–
14	69	133	–	–
15	73	122	–	–
16	63	110	–	–
17	71	130	–	–
18	70	114	–	–
19	74	126	–	–
20	69	125	–	–
21	64	127	–	–
22	73	118	–	–
23	73	120	–	–
24	73	115	–	–
25	77	127	–	–
26	66	117	–	–
27	73	136	–	–
28	69	135	–	–
29	63	145	–	–
30	76	129	–	–
Mean (ME)	71.2 (±3.75)	128.9 (±9.08)	–	–

All measurements are given in micrometers (μm).

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2024.e00237.

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