Parasitology Research (2021) 120:145–152
https://doi.org/10.1007/s00436-020-06951-6

HELMINTHOLOGY - ORIGINAL PAPER

Alaria alata mesocercariae prevalence and predilection sites in amphibians in Latvia

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Received: 3 April 2020 / Accepted: 28 October 2020 / Published online: 18 November 2020

Abstract

Alaria alata is known as a trematode with a complex life cycle. The trematode *Alaria alata* infects amphibians as second intermediate hosts. In the present study, we examined 390 amphibians—European water frogs *Pelophylax esculentus* complex ($n = 335$), common frogs *Rana temporaria* ($n = 19$), moor frogs *Rana arvalis* ($n = 3$), and common toads *Bufo bufo* ($n = 30$) collected from randomly selected wetland habitats in Latvia. Out of all examined specimens, 80 were tadpoles and 310 were adult amphibians. Mesocercariae of *A. alata* was detected in 108 specimens from all examined amphibian species, except the common toad, reaching the overall prevalence of 27.7%. Tadpoles were found to be more frequently infected with *A. alata*, when compared with adults, 58.8% and 22.4%, respectively. The results showed that mesocercariae accumulate in visceral membranes, different internal organs, and muscles in the head area. This is a comprehensive study to identify *A. alata* mesocercariae predilection sites in amphibians.

Keywords Pelophylax · Frogs · Predilection sites · Mesocercariae

Introduction

Alaria alata is known as a common trematode in European canids. The adult trematode lives in the small intestine of definitive hosts. Some hosts, however, are able of harbor mesocercariae, including felids, mustelids, and procyonids (Szczęsna et al. 2008; Möhl et al. 2009; Castro et al. 2009; Tăbăran et al. 2013; Rentería-Solis et al. 2013; Takeuchi-Storm et al. 2015; Rodríguez-Ponce et al. 2016; Martinković et al. 2017; Ozoliņa et al. 2018; Ozoliņa et al. 2019). Scant information is available on the freshwater snail (1st intermediate host) and the amphibian (2nd intermediate host) roles in the *A. alata* life cycle (Shimalov and Shimalov 2000, 2001; Shimalov et al. 2000, 2001; Portier et al. 2012; Chikhlyaev and Ruchin 2014; Chikhlyaev et al. 2016; Patrelle et al. 2015; Voelkel et al. 2019; Huguenin et al. 2019). Therefore, more attention has been paid to paratenic hosts as potential reservoirs of alariosis. More frequently, wild boars were the object of study, and *A. alata* mesocercariae prevalence varied from 1.6 to 11.5% in Western Europe (Riehn et al. 2012; Paulsen et al. 2012, 2013; Berger and Paulsen 2014; Malešević et al. 2016). At the same time, significantly higher prevalence (44.3%) was observed in north-eastern Poland (Strokowska et al. 2020).

Amphibians are second intermediate hosts of the trematode. The mesocercariae can localize in different tissues of intermediate and paratenic hosts in many species of mammals and also, potentially, in humans (Skrjabin 1960; Borgsteede 1984; Shimalov and Shimalov 2000, 2001, 2003; Shimalov et al. 2000, 2001; Segovia et al. 2003; Craig and Craig 2005; Möhl et al. 2009). Different development stages of

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amphibians became infected when the *A. alata* cercariae actively penetrated their skin with a specific penetration apparatus after cercariae were released from the freshwater snail (1st intermediate host) and actively sought their next host in an aquatic environment (Galaktionov and Dobrovolskij 2003; Möhl et al. 2009; Portier et al. 2012). Cercariae that penetrate the 2nd intermediate host tissues develop to an additional mobile larval stage, mesocercariae, instead of developing into an encysted metacercaria which was typical in other trematodes (Pearson 1956; Möhl et al. 2009). Experimental studies with *A. alata* have not been done; however, North American *Alaria* species showed that mesocercariae developed in the second intermediate host tissues within 2 weeks (reviewed by Olsen 1974).

In Europe, several studies have shown the *A. alata* mesocercariae occurrence in different hosts. In particular, the prevalences of *A. alata* in amphibians and reptiles from Belarus were studied in the smooth newt *Triturus vulgaris* (10.7%), the northern crested newt *Triturus cristatus* (20.0%), the sand lizard *Lacerta agilis* (17.0%), the viviparous lizard *Lacerta vivipara* (9.1%), the grass snake *Natrix natrix* (21.2%), the smooth snake *Coronella austriaca* (20.0%), the adder *Vipera berus* (22.6%), the common toad *Bufo bufo* (8.0%), the natterjack *Bufo calamita* (36.4%), and the green toad *Bufo viridis* (67.9%) (Shimalov and Shimalov 2000, 2001; Shimalov et al. 2000, 2001). By gathering data over the past 30 years and supplemented with their own original study results, researchers found *A. alata* mesocercariae from Volga Basin, Russia in both the common brown frog, and the European common toad (Chikhlyaev and Ruchin 2014; Chikhlyaev et al. 2016). The previous studies in Europe had shown that *A. alata* mesocercariae might be located in different body parts of a frog, including its body cavity, internal organs, head, periorbital, hindlimb, and forelimb. The prevalence of *A. alata* mesocercariae ranges from 39% in the brown frog group (e.g., *Rana dalmatina* and *R. temporaria*) to 87% in the water frog group (Patrelle et al. 2015; Voelkel et al. 2019).

The aim of the present study was to estimate the prevalence of *A. alata* mesocercariae and to determine their predilection sites in amphibians in Latvia.

Material and method

During the period 2017–2019, amphibians were collected with a special permission that was granted by the Latvian authorities for the collecting and euthanizing of amphibians for scientific purposes (26/2017-E, 14/2018-E, 21/2019-E – Nature Conservation Agency of Latvia).

Overall, 390 specimens were collected in 86 different randomly selected wetlands (Fig. 1), permanently full of water. The geographical coordinates of the collection sites were converted to a map layer and plotted on a base map of Latvia (divided into planning regions) using a Geographic Information System (GIS) Program, the ArcGIS version 10 (ESRI 2011).

Adult frogs and tadpoles were gathered from shallow portions using a standard O-frame net with a diameter of 0.6 m, 5-mm mesh size and a handle length of 1.5 m. The collected samples were placed in a disposable box with water (300 ml) and transported to the laboratory within 8 h and kept at + 4 °C until further procedures. Euthanasia was performed in the laboratory by a blow to the head as per European Union requirements and the Federation of European Laboratory Animal Science Association regulations (FELASA) (Guillen 2012), under the supervision of a FELASA-certified specialist.

The species of all collected amphibians and tadpoles were identified based on morphological criteria (Bannikov et al. 1977) as the European common frog *Rana temporaria*, the European moor frog *Rana arvalis*, the European common toad *Bufo bufo*, and the water frog group *Pelophylax esculentus* complex (*Pelophylax ridibundus*, *Pelophylax lessonae*, and the hybrid *Pelophylax esculentus*). The development stage (adult frog or tadpole) and adult sex were noted. Weight (g) was measured using calibrated scales with precision ± 0.01 g. Body length (cm) was measured from the nose to the cloaca.

Amphibian skin was peeled off and divided into two parts (head and torso) and rinsed with distilled water. Next, the head, torso, internal organs (lungs, liver, kidneys, and intestinal wall) and their visceral membranes, forelimbs, and hindlimbs musculature were examined by using the compression method (Justine et al. 2012; Khalil et al. 2014). Dissected parts were compressed between two slides and analyzed with a stereomicroscope. All mesocercariae were separated from the muscle tissue and analyzed with a microscope (× 100–× 400). *Alaria alata* mesocercariae identification was based on morphological characteristics, such as the number of glandular cells, body shape, and size (Skrjabin 1960; Möhl et al. 2009; Patrelle et al. 2015). All of the found mesocercariae met the criteria and were identified as *Alaria alata*.

For each amphibian species, the prevalence (percentage of infected animals from all analyzed animals), median intensity (median number of a parasite considering only the infected members of said host species), and mean intensity (average number of a parasite considering only the infected animals of the host species) of *A. alata* mesocercariae were calculated (Bush et al. 1997; Reiczigel et al. 2019). Confidence intervals (95%) of prevalence were calculated using the Clopper-Pearson method (Clopper and Pearson 1934). Confidence level of median was reported as the shortest interval that reaches the desired confidence level (Bush et al. 1997; Carpenter and Bithell 2000). The differences of mesocercariae prevalence and medians between species, age groups, sex, and predilection sites were analyzed with the Fisher's exact test and Mood's median test (Sen 2005; Reiczigel et al. 2008). Statistical analyses were performed using R (R Core Team 2019).

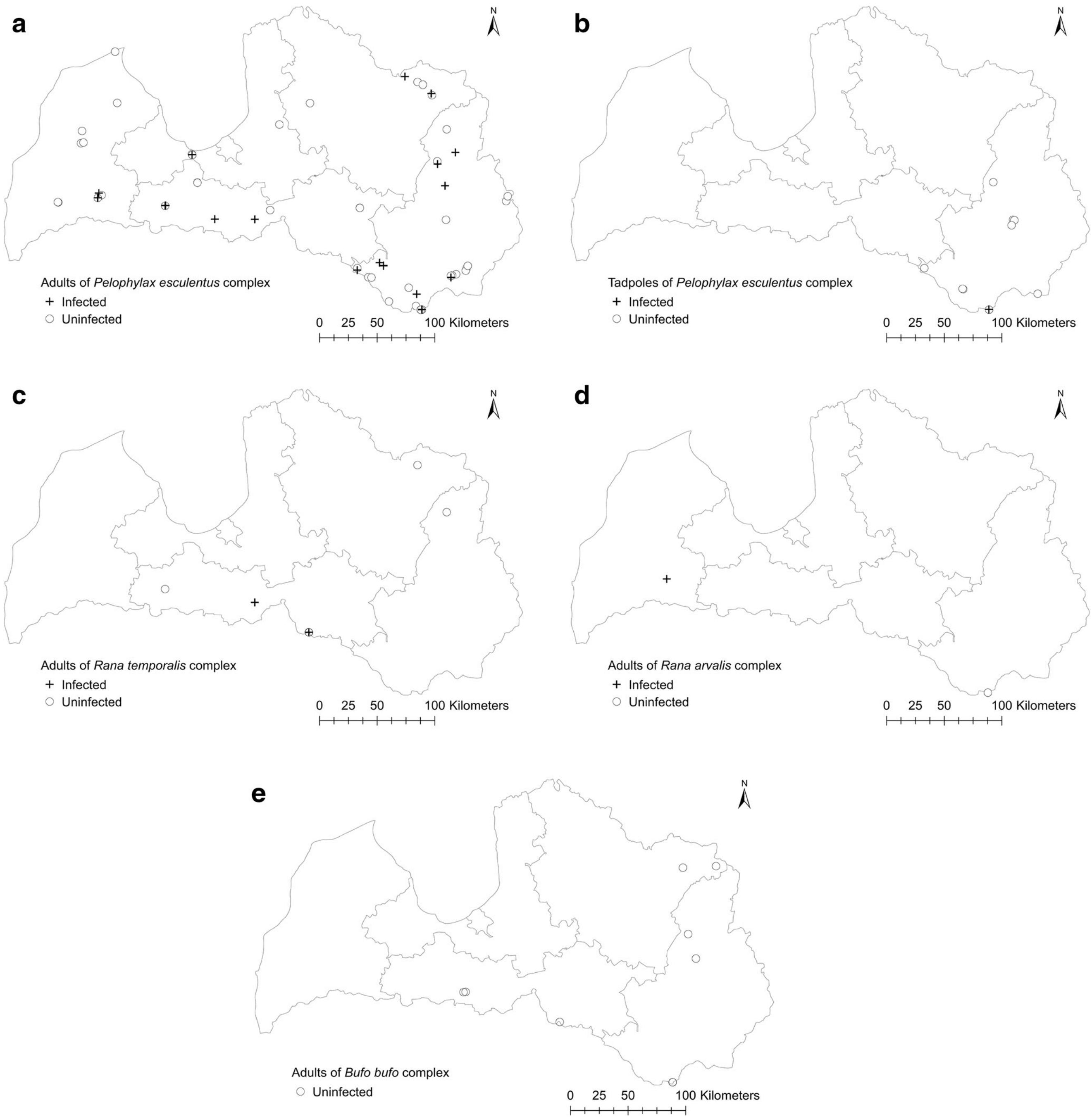

Fig. 1 Amphibian collection sites from 2017 to 2019. **a** Adults of European water frogs *Pelophylax esculentus* complex; **b** tadpoles of European water frogs *Pelophylax esculentus* complex; **c** adults of common frogs *Rana temporaria*; **d** adults of moor frogs *Rana arvalis*; and **e** adults of common toads *Bufo bufo*

Results

In total, *A. alata* mesocercariae were detected in 28 of 86 (32.6%; CI 95% 23.6–43.1) randomly selected wetlands (Fig. 1). Mesocercariae of *A. alata* were found in all analyzed frog species, while none of the analyzed common toads were found to be infected (Table 1). There were no significant differences observed between the prevalence in different host species. Nonetheless, 108 of the 390 amphibians examined for *A. alata*, including tadpoles, were infected with an overall prevalence of 27.7% (CI 95% 23.5–32.3). From all collected specimens, including tadpoles ($n = 80$), the highest (85.9%; $n = 335$) was from the water frog group.

Table 1 The prevalence of *Alaria alata* mesocercariae in different amphibian species (adults and/or tadpoles) in Latvia

Species	Developmental stage	No. of investigated /positive animals	Prevalence, CI 95%	Mesocercariae mean intensity ± SD (range)	Mesocercariae median intensity (median CL)
European water frog sensu *lato* group	Tadpole	80/47	58.8 (47.5–69.6)	8.7 ± 16.3 (1–95)	4 (96.0% 2–5)
Pelophylax esculentus complex	Adult	255/57	22.4 (17.4–28.0)	28.9 ± 54.3 (1–237)	4 (96.8% 3–8)
Common frog *Rana temporaria*	Adults	19/3	15.8 (3.4–39.6)	20.0 ± 15.7 (6–37)	N/A
Moor frog *Rana arvalis*	Adults	3/1	33.3 (0.8–90.6)	2.0 ± N/A (2)	N/A
Common toad *Bufo bufo*	Adults	33/0	N/A	N/A	N/A

N/A not applicable

Overall, from 2120 observed mesocercariae, 7.0% (95% CI 6.0–8.2%) were encysted (Fig. 2).

Notably, only tadpoles from the water frog group were analyzed, and the *A. alata* mesocercariae prevalence in tadpoles was significantly higher ($p < 0.0001$) than in adults from the same water frog group. Additionally, no significant differences between median intensity in different amphibian development stages were observed.

Sex was determined for 210 of 255 adult water frogs and 18 of 19 common frog adults (Table 2). Still, no significant differences ($p > 0.05$) were observed between the prevalence and median mesocercariae intensity and different sexes, although a tendency of higher mesocercariae median intensity in males of the water frog group was observed.

Overall weight (0.3–80.0 g) and size (1.1–10.5 cm) were determined for 240 of 255 adult water frogs. When the abundance of *A. alata* mesocercariae was analyzed, a slight linear tendency was detected with weight ($r = 0.19$, $p = 0.0034$) and size ($r = 0.22$, $p = 0.0004$).

Our study has shown that *A. alata* mesocercariae predilection sites in adult frogs were the head, internal organs, and visceral membranes (Table 3). Moreover, mesocercariae in tadpoles were found predominantly in the head and torso regions. Significantly higher prevalence was observed in the tadpole head ($p < 0.0001$) and torso ($p < 0.0001$) compared with the adults of the same species and sites. Most often, mesocercariae in adult frogs were observed in both the head and visceral membrane (64.7%, CI 95% 41.2–82.8), while mesocercariae in tadpoles were mostly detected in both the head and torso (55.0%, CI 95% 34.2–74.19). Internal organs were analyzed separately, and significantly higher ($p = 0.005$) median intensity was observed in the livers of water frogs. Yet, due to the size of the tadpole, it was not possible to distinguish the internal organs. Furthermore, in one specimen of an adult common frog, mesocercariae were found in the intestinal wall. Overall, 1487 of 2120 mesocercariae (70.1%, CI 95% 68.2–72.1) were detected in the body cavity—925 (43.6%; CI 95% 41.5–45.8) in the internal organs and 562 (26.7%; CI 95% 24.7–28.4) in the visceral membranes.

Discussion

This study shows a high prevalence of *A. alata* mesocercariae in the water frog group. Common frogs and moor frogs indicated that these amphibians were suitable intermediate hosts

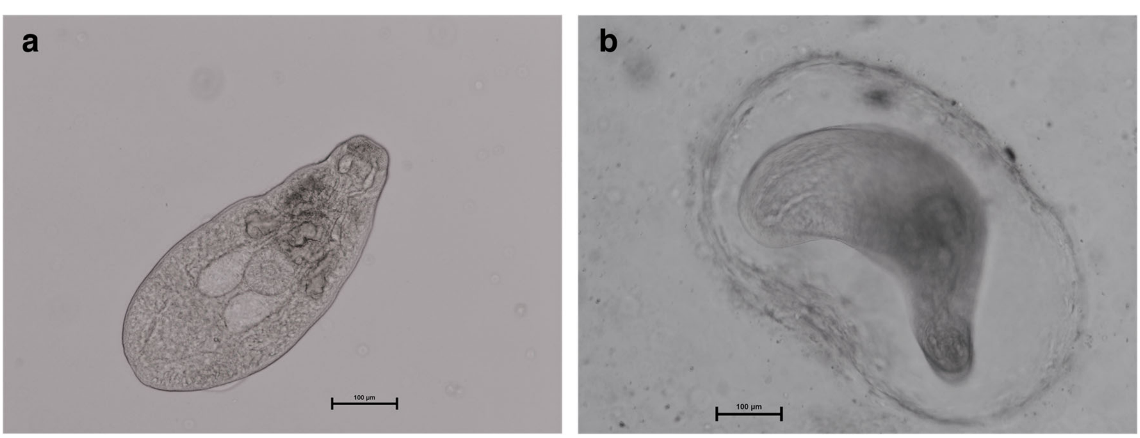

Fig. 2 **a** *Alaria alata* mesocercariae and **b** *A. alata* mesocercariae encysted, isolated from second intermediate host

Table 2 *Alaria alata* mesocercariae occurrence in different sexes in Latvia

Species	Sex	No. of analyzed/infected animals	Prevalence, CI 95%	Mesocercariae mean intensity ± SD (range)	Mesocercariae median intensity (median CL)
European water frog sensu *lato* group *Pelophylax esculentus complex*	Female	100/26	26.0 (17.7–35.7)	27.7 ± 52.3 (1–201)	3 (95.8% 2–18)
	Male	110/26	23.6 (16.1–32.7)	28.3 ± 53.1 (1–237)	5.5 (96.2% 3–12)
Common frog *Rana temporaria*	Female	12/2	16.67 (2.1–48.4)	27.0 ± 14.1 (17–37)	N/A
	Male	6/1	16.67 (0.4–64.1)	6 ± N/A (6)	N/A

N/A not applicable

for *A. alata*, while none of the analyzed common toads was found to be infected in Latvia. In the previous studies, common toads had been found to be suitable intermediate hosts for *A. alata* as well. For instance, mesocercariae were observed in 2 out of 25 (0.8%) common toads from Belorussian Polesie (Shimalov and Shimalov 2001). Also, in the Volga Basin, in Russia, the common toad was found to be a suitable second intermediate host for *A. alata* (Chikhlyaev et al. 2016). In the present study, the analyzed number of common toads was limited ($n = 31$), and likely the prevalence and median intensity of *A. alata* mesocercariae could have been underestimated.

In the present study, prevalence of *A. alata* mesocercariae in common frogs and moor frogs was 15.8% ($n = 19$) and 33.3% ($n = 3$), respectively. The prevalence of *A. alata* mesocercariae in common frogs can reach 27.0% ($n = 37$) (Patrelle et al. 2015), though there are no previously published data on prevalence in moor frogs. Nevertheless, the adult water frog group analysis showed similar results compared with our study, 17.3% ($n = 29$) and 22.4% ($n = 225$), respectively (Patrelle et al. 2015). All investigated frog species spawn from April to May and only water frogs inhabit the aquatic environment all the time, while common and moor frogs are

Table 3 Predilection sites of *Alaria alata* mesocercariae in different frog species and their development stages in Latvia

Species	Localization	No. of infected samples	Proportion, CI 95%	Mesocercariae mean intensity ± SD (range)	Mesocercariae median intensity (median CL)
European water frog group *Pelophylax esculentus* complex adults ($n = 255$)	Head	21	8.2 (5.2–12.3)	8.8 ± 14.2 (1–52)	4 (96.1% 1–6)
	Periorbital	7	2.7 (1.1–5.6)	3.7 ± 2.2 (1–8)	3 (98.4% 1–8)
	Torso	1	0.4 (0–2.2)	2.0 ± N/A (2)	NA
	Visceral membrane	30	11.8 (8.1–16.4)	17.5 ± 36.5 (1–189)	3 (96.2% 1–5)
	Internal organs (total)	26	10.2 (6.8–14.6)	35.0 ± 50.0 (1–201)	17.5 (96.2% 3–35)
	Internal organs (divided):				
	Lungs	1	0.4 (0–2.2)	8.0 ± N/A (8)	NA
	Liver	10	3.9 (2.1–7.2)	62.1 ± 62.9 (3–201)	37.5 (97.9% 19–142)
	Kidneys	1	0.4 (0–2.2)	24.0 ± N/A (24)	NA
	Intestinal wall	21	8.2 (5.4–12.3)	14.7 ± 21.1 (1–89)	4 (95.7% 3–17)
European water frog group *Pelophylax esculentus* complex tadpoles ($n = 80$)	Head	32	40.0 (29.2–51.6)	6.3 ± 9.9 (1–54)	4 (96.5% 2–5)
	Periorbital	4	5.0 (1.4–12.3)	1.0 ± 0.0 (1–1)	NA
	Hindlimb	4	5.0 (1.4–12.3)	2.0 ± 1.4 (1–4)	NA
	Torso	23	28.7 (19.2–40.0)	7.0 ± 17.7 (1–87)	2 (95.3% 1–3)
	Visceral membrane	7	8.7 (3.6–17.2)	3.4 ± 4.0 (1–11)	1 (98.4% 1–11)
	Internal organs (total)	2	2.5 (0.3–8.7)	4.5 ± 4.9 (1–8)	NA
Moor frog *Rana arvalis* ($n = 3$)	Head	1	100 (16.75–100)	2.0 ± N/A (2)	NA
Common frog *Rana temporaria* ($n = 19$)	Head	2	10.5 (1.7–32.6)	21.0 ± 21.2 (6–36)	NA
	Visceral membrane	2	10.5 (1.7–32.6)	6.0 ± 7.1 (1–11)	NA
	Internal organs (total)	1	5.3 (0.0–26.5)	6.0 ± N/A (6)	NA

N/A not applicable

present in water only when spawning. These frogs mostly prefer stagnant waters where only a few snails have the capacity to infect the frogs in a waterbody (Portier et al. 2012; Patrelle et al. 2015).

The presence of *A. alata* mesocercariae in the intermediate and paratenic hosts as a facultative part of a parasite's life cycle increased transmission opportunities. In this parasite development stage, *A. alata* could survive several host transitions unharmed (Möhl et al. 2009). Experimental studies with *A. arisimoides* and *A. canis* cercariae showed that amphibians could get infected in two ways: via cercariae penetration through the skin and/or via cannibalism of other amphibians infected with mesocercariae (Pearson 1956). According to the previous studies, depending on infection route, amphibians could act as second intermediate hosts if the infection occurred with cercariae penetrations or paratenic hosts if infection occurred via consumption of other infected amphibians (Pearson 1956; Patrelle et al. 2015). Cannibalism has been reported for Ranidae frogs, and it could be observed in several other frog species, especially in cases where there were dense populations of tadpoles (Ruchin and Ryzho 2002; Covaciu-Marcov et al. 2005; Mollov et al. 2010). The changes in the ecological conditions in the habitat and the increased growth of population could force specimens toward cannibalism as cannibalism emerges as a mechanism to increase the survival rate of amphibians (Crump 1992; Stebbins and Cohen 1995).

In the present study, 7.0% of detected mesocercariae in frogs were encapsulated and located mostly in the visceral membrane. Several previous studies had also mentioned the presence of similar pseudocysts (Tăbăran et al. 2013; Patrelle et al. 2015; Uhrig et al. 2015). Similar to other cysts, they are circular and encapsulated, but are not lined by a functional epithelium. The mucus around the mesocercariae was most likely produced by the host; inflammation was the result of direct tissue damage rather than an immune reaction targeted toward the parasitic antigens (Tăbăran et al. 2013; Uhrig et al. 2015).

After being ingested, *A. alata* mesocercariae could migrate through the intestinal wall and circulate to the muscle tissues and different organs (Möhl et al. 2009), while Pearson (1956) observed that regardless the route of infection, mesocercariae in frog tissues distribute equally, in the muscle tissues ventrally in the distal part of the thighs or between the sternum and the hyoid. In the current study, the following predilection sites were determined for *A. alata* mesocercariae in amphibians: the head, especially periorbital muscles, torso, especially under skin, internal organs, including the lungs, liver, kidneys, intestinal wall, and organ visceral membranes. Although *A. alata* mesocercariae were mostly found in the head, including periorbital region, these results correspond with the previous studies, which also had reported head, hindlimbs, and torso as a predilection sites for *A. alata* mesocercariae in amphibians (Patrelle et al. 2015; Voelkel et al. 2019). The highest mesocercariae intensity in the tissues around the eyes could possibly lead to impaired vision of frogs and tadpoles and, thus, make them more susceptible to predators assuring *A. alata* transmission (Patrelle et al. 2015).

The present study is the first one that shows no differences in *A. alata* mesocercariae prevalence in either the water frog group or in the common frogs between different sexes. An earlier study comparing the pattern of the other parasite infection and the amphibian host sex also did not show any differences (Hamann et al. 2006). It should be noted, however, that there is no notable difference in behavior or biology in tadpoles of different sex of these two species because these differences only become apparent once the amphibians sexually mature into adults (Lannoo 2005).

Significant difference was observed between *A. alata* mesocercariae prevalence in tadpoles and adult frogs. Tadpoles probably have higher risk of infection due to their activity and easy access through the thin skin; the epidermis is much thinner, compared with adult frogs which have a thicker, harder outer skin (Pearson 1956). Still, in a previous study where 23 tadpoles and 29 adults of water frog group were analyzed, no significant differences between the frogs' developmental stages were observed (Patrelle et al. 2015).

To our knowledge, this is the most comprehensive study on *A. alata* mesocercariae occurrence and their predilection sites in amphibians not only in the Baltic region but also in all of Europe. Present study shows that *A. alata* is common in various species of amphibians in Latvia, and it indicates the gaps of knowledge regarding potential *A. alata* 2nd intermediate hosts in different regions in Europe.

Acknowledgments We thank Daina Pūle for her valuable work in preparing maps for this study. This study was conducted under the auspices of the project: "The biological and ecological triggers causing the expansion of the invasive fish: the Chinese Sleeper (*Perccottus glenii*), in Eastern Europe" (Latvian – Ukrainian project Nr. LV-UA/2018/6).

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