

# Prevalence and intensity of *Alaria alata* (Goeze, 1792) in water frogs and brown frogs in natural conditions

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**Abstract** In the last 15 years, the mesocercariae of *Alaria alata* have frequently been reported in the wild boar during routine *Trichinella* inspections made compulsory for the trade of venison meat in Europe. If these studies have focused primarily on mesocercariae isolated from meat, few works have been done so far to understand the circulation of the parasite in natural conditions especially in the intermediate hosts. This study focuses on the second intermediate hosts of this parasite assessing the suitability of two amphibian groups—brown frogs and water frogs *sensu lato*—for mesocercarial infection on an area where *A. alata* has already been identified in water snails and wild boars. During this study, both groups showed to be suitable for mesocercarial infection, with high prevalence and parasite burdens. Prevalence was higher in the brown frog group (56.9 versus 11.54 % for water frogs) which would indicate that it is a preferential group for infection on the study area, though reasons for this remain to be investigated. No significant difference among prevalences was observed between tadpoles and frogs. This study, the first focusing on *A. alata* in these amphibians in Europe, provides further information on circulation of this parasite in natura.

**Keywords** *Alaria alata* · Mesocercariae · Water frog · Brown frog

## Introduction

In Europe, the implementation in 2005 of compulsory *Trichinella* search for every hunted wild boar (*Sus scrofa*) entering commercial circuits lead to the frequent highlighting of the mesocercarial stage called *Distomum musculorum suis*, Duncker 1896 of a diplostomid trematode—*Alaria alata* (Goeze, 1782). The mesocercariae stage was identified for the first time in 1881 by Duncker in the muscles of pigs (Duncker 1896; Leuckart 1901) and was subsequently observed in wild boars in Europe with non-negligible prevalences and parasite burdens in specific areas (Wójcik et al. 2001; Milesevic et al. 2004; Möhl et al. 2009; Riehn et al. 2012; Paulsen et al. 2012, 2013; Széll et al. 2013). The first report in French boars was published in 1953 by Dollfus and Chabaud (1953) and no further mention appears after, before its recent observations in 2003 (Portier et al. 2011, 2014). The cycle of the species of this genus has two intermediate hosts, a snail (Ruszkowski 1922; Bosma 1931; Pearson 1956; Nikitina 1986; Wójcik et al. 2001; Portier et al. 2012) and an amphibian (Skrjabin 1965; Shimalov and Shimalov. 2001; Shimalov et al. 2001a; Shimalov 2002; Andreas 2006). The paratenic hosts for the mesocercarial stage recognized in Europe for *A. alata* belong to different vertebrate hosts especially those eating amphibians (*S. scrofa*, *Procyon lotor*, mustelids, small mammals, brown bear, birds, and reptiles) (Brumpt 1945; Skrjabin 1965; Shimalov et al. 2000, 2001b; Riehn et al. 2012; Tabaran et al. 2013; Renteria-Solis et al. 2013; Portier et al. 2014). The metacercarial and adults stages respectively parasitize lung and

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small intestine of canids such as foxes (*Vulpes vulpes*), raccoon dogs (*Nyctereutes procyonoides*) (Al-Sabi et al. 2013), wolves (*Canis lupus*) (Shimalov et al. 2000; Moks et al. 2006), golden jackal (*Canis aureus*) (Cirovic et al. 2015), and the domestic dog is a potential definitive host (Stefanski and Tarczyski 1953; Savinov 1953; Umur 1998).

Bibliographical investigations revealed that American *Alaria* species were zoonotic parasites with documented, sometimes fatal, human cases (Lester and Freeman 1975; Fernandes et al. 1976; Freeman et al. 1976; McDonald et al. 1994) and that no biological reason could exclude the same potential for the European *A. alata* mesocercaria, non-human primates acting as paratenic hosts according to the consequence of experimental infection in a rhesus monkey (Odening 1963).

Humans become infected through eating mesocercaria in raw or undercooked game (wild boar) or frog meat (Fried and Abruzzi 2010). In the last 10 years, several research teams have focused on game meat qualities and safety, trying out new detection methods (Riehn et al. 2010, 2013), assessing its resistance to thermal and chemical treatments (González-Fuentes et al. 2014a, b, 2015), and spectrum of paratenic and definitive hosts (Castro et al. 2009; Li et al. 2013; Möhl et al. 2009; Murphy et al. 2012; Riehn et al. 2012; Al-Sabi et al. 2013; Paulsen et al. 2013; Renteria-Solis et al. 2013; Tabaran et al. 2013; Portier et al. 2014; Phan et al. 2015). However, only one study has focused on amphibian meat in the USA (Fried and Abruzzi 2010), and none in Europe, despite the fact that these animals are still a traditional meal in several European countries—hindlimb of brown frogs and water frogs are currently consumed in some Regions, exported alive from the Balkanic region, Egypt, and Turkey, or coming from French frog breeding farms (Pagano et al. 2003; Neveu 2004; Schmeller et al. 2007; Holsbeek et al. 2008)—and that several human cases in the USA were contracted after eating amphibians (Fernandes et al. 1976; Beaver et al. 1977; McDonald et al. 1994).

Since the last investigations made to elucidate the parasite's complete life cycle in the late 1930s, only few recent studies have focused on its circulation in aquatic snails in natural conditions (Wójcik et al. 2001; Portier et al. 2012), but amphibian were barely studied (Shimalov and Shimalov 2001; Shimalov et al. 2001a; Shimalov 2002; Andreas 2006; Lukijanov et al. 2008). Therefore, very little knowledge exists on the nature of mesocercarial presence in amphibians, parasite burdens, and preferential species for infection.

In this study, we investigated the role of the two most common amphibian groups in Europe, the water frogs and the brown frogs *sensu lato*, as second intermediate hosts of *A. alata* in natural conditions. Both groups have already been shown as susceptible to mesocercarial infection. In addition, the interrelationship between aquatic snails and amphibians through the transmission of *A. alata* mesocercaria was observed.

## Material and methods

Brown frogs and water frogs were collected from two closed sites—the “Coulon canal” and the “Argentolle pond woods”—on the Der-Chantecoq area located in the Northeast of France in the Marne Department (4°45'E, 48°35'N), a place already known for the presence of the parasite *A. alata* in snails (Portier et al. 2012). Both sites are characterized by shallow waters, a gentle slope, and dense vegetation, but “Coulon canal” is always full of water with a slow flowing stream (3 m wide and 60 cm deep in its center) bordered by reeds, whereas Argentolle is a floodable grassy marsh with small temporary ponds. We obtained a permit granted by the relevant french authorities allowing us to collect amphibians during their breeding seasons by hand or using a deep net, through eight collecting campaigns between April 2011 and July 2012. All collected amphibians were brought to the laboratory and kept at +4 °C until testing. Amphibians were split into two developmental stages: tadpoles or frogs (including metamorphs called “froglets,” juveniles, sub-adults, and reproductive adults). Identification of each amphibian was based on morphological criteria as belonging to the brown frog *sensu lato* group (e.g. *Rana dalmatina* or *Rana temporaria*) or to the water frog *sensu lato* group (*Pelophylax ridibundus*, *Pelophylax lessonae*, and the hybrid *Pelophylax esculentus*) (Duguet and Melki 2003; Muratet 2007). Moreover, molecular analysis were performed on water frogs for which it was possible to keep a small part of tissue in 95° ethanol, according a PCR-RFLP method developed by Patrelle et al. (2011) to realize a reliable taxonomic identification among this group. Collected amphibians were euthanized by cerebral elongation and finely dissected under stereomicroscope. Remaining tissues after dissections were analyzed according to a modified Baermann technique usually used for the detection of pulmonary protostrongylids larvae (Baermann 1917): tissues were put to decant in a sieve reposing on a stand-glass and immersed in water at 46 °C for 30 mn in order to retrieve any remaining parasites. All parasites were counted and collected and then preserved in 95° ethanol for molecular analysis. Molecular identification of some *A. alata* mesocercaria from amphibians was carried out using the same methods as described by Portier et al. (2011) to confirm morphological identifications of the specimens observed fresh, especially the differential diagnosis with mesocercariae of Strigeidae (Dubois 1968).

Parasitic prevalences according to frog's groups, taxa among water frogs, sites, and age classes were compared using a Pearson's chi-square test. Parasitic burdens were analyzed using a Wilcoxon Man Whitney procedure, with the Wilcoxon rank-sum test (Crawley 2007). All statistical analyses were performed using R software (The R Foundation for Statistical Computing 2005).

## Results

A total of 59 amphibians among the 150 individuals analyzed were found positive for the presence of *A. alata* mesocercaria (Table 1). These mesocercaria were free, mobile, but also encapsulated alone or sometimes in pairs within the amphibians' tissues (Fig. 1). These capsules were transparent, fragile, and barely adhered the hosts' tissues. During the autopsies of tadpoles and frogs, we found on fresh materials others parasites, especially some "mesocercaria like" (same shape; smaller size after fixation: 480 versus 680  $\mu\text{m}$ ) differ from those of *Alaria* spp. by the number of glandular cells and close to the mesocercarial stage of *Strigea* (Fig. 1). The two samples (corresponding to the Fig. 1c, d) were sequenced on the D2 domain of the 28S, and we obtained two sequences clearly different for the one of *A. alata*. The sequences, closed to the taxa *Strigeidae*, were deposited in Genbank (numbers KT362372 and KT362373).

For water frogs *sensu lato*, 52 individuals—23 tadpoles and 29 adult—were collected and analyzed for the presence of *A. alata*, among which 6—1 and 5, respectively—harbored from 6 to 314 mesocercaria, with an average parasitic burden of 83.6 mesocercaria (SD=131.04; Table 1 and Fig. 2). Taxonomic identification was realized on 31 individuals, and revealing that the three taxa of water frogs are present in Der-Chantecoq area. Indeed, 7 *P. lessonae*, 3 *P. ridibundus*, and 21 hybrids *P. esculentus* were identified; 3 hybrids and 2 *P. lessonae* were parasited by *A. alata*, and we observed no significant difference of prevalence between taxa ( $\text{Chi}^2=1.4308$ ,  $df=2$ ,  $p$  value=0.5).

Moreover, 98 brown frogs *sensu lato* were collected composed of 61 tadpoles and 37 adult. *A. alata* mesocercaria were

observed among 53 individuals (33 tadpoles and 20 adult) in a range from 1 to 331, with in global an average parasitic burden of 65.48 mesocercaria (SD=90.16), 86.75 (SD=96.54), and 18.67 (SD=20.81), respectively, for tadpoles and for frogs (Table 1).

In general, brown frogs were more parasited by *A. alata* than water frogs ( $\text{Chi}^2=24.016$ ,  $df=1$ ,  $p<0.001$ ). Indeed, this is true for both tadpoles ( $\text{Chi}^2=10.986$ ,  $df=1$ ,  $p=0.009$ ) and adult ( $\text{Chi}^2=4.806$ ,  $df=1$ ,  $p=0.028$ ; Fig. 2). There is no significant difference of parasitic burdens between water frogs and brown frogs ( $W=96.5$ ,  $df=1$ ;  $p=0.484$ ).

Regarding the development stage effect, we noted that there is no significant difference between tadpoles and frogs, which have nearly the same parasitic prevalence for *A. alata* ( $\text{Chi}^2=0$ ,  $df=1$ ,  $p=1$ ) and also the same parasitic burden ( $W=295$ ,  $df=1$ ,  $p=0.248$ ).

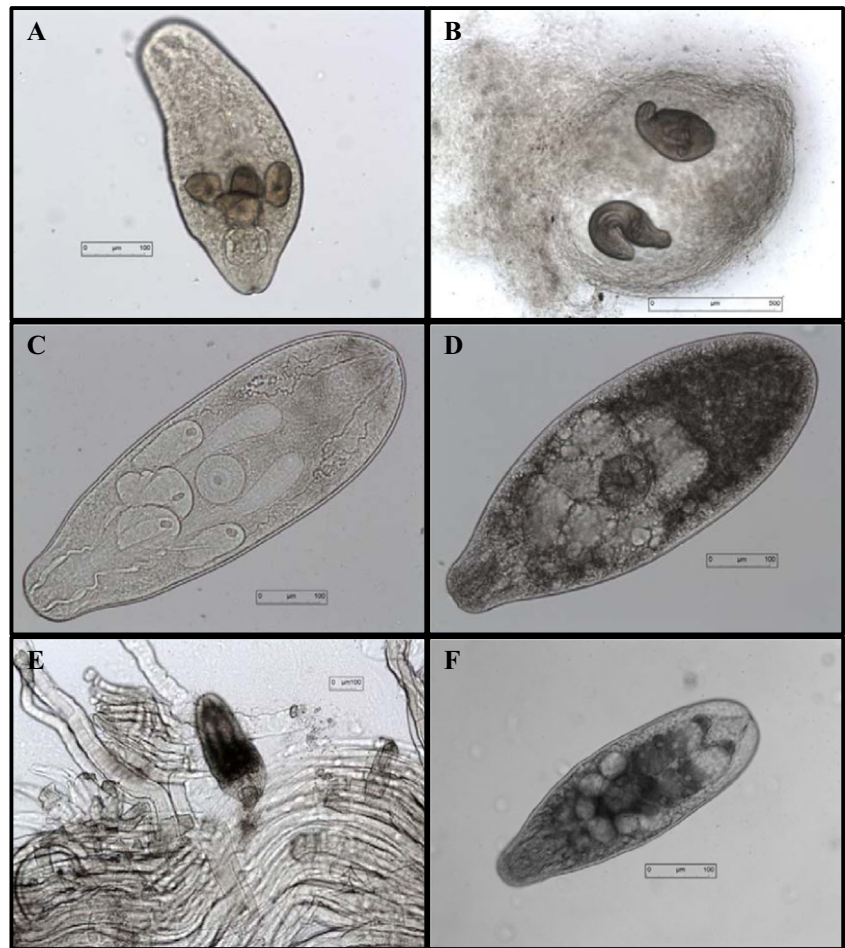
We observed an effect of the sampled site, since all amphibians collected in Argentolle are significantly more parasited by *A. alata* than those from Coulon ( $\text{Chi}^2=6.525$ ,  $df=1$ ,  $p=0.011$ ; Table 1). Among water frogs, we observed the same trend ( $\text{Chi}^2=5.621$ ,  $df=1$ ,  $p=0.018$ ), but not for brown frogs for which the prevalence for *A. alata* is nearly the same whatever the origin ( $\text{Chi}^2=0.035$ ,  $df=1$ ,  $p=0.852$ ). However, if amphibians from Argentolle are more often parasited, their parasitic burden is significantly less important than amphibians from Coulon ( $W=151$ ,  $df=1$ ,  $p<0.001$ ).

The presence of *A. alata*'s mesocercaria was observed in all parts of the body. However, the parasite appears to have preferential tissue localization in frogs since more mesocercaria seemed to be concentrated in the head, and especially in periorbital tissues, representing up to a third of total infection (Table 2). Encapsulated forms were observed in diverse localizations: insertion of the mandible, periorbital tissues, around the sternum, the haunch, and the posterior limbs.

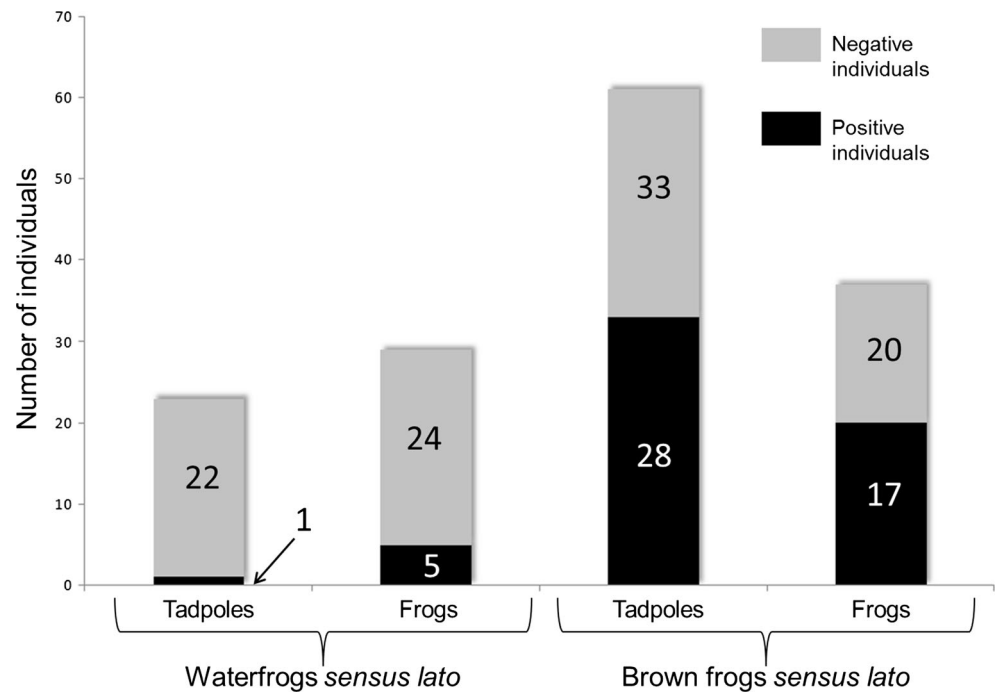
**Table 1** Number of tadpoles and frogs parasited by *A. alata* according their species *sensu lato*, and their collection site

Species	Site	Developmental stage	Nb collected	Effect if harboring <i>A. alata</i>	Parasite burdens
Water Frogs <i>sensu lato</i>	Coulon	Tadpoles	20	1	6
		Adults	21	1	6
	Argentolle	Tadpoles	3	0	–
		Adults	8	4	12 to 314
		Total	52	6	
Brown frogs <i>sensu lato</i>	Coulon	Tadpoles	30	19	9 to 280
		Adults	18	6	1 to 56
	Argentolle	Tadpoles	31	14	2 to 15
		Adults	19	14	1 to 331
		Total	98	53	
Total of Amphibians			150	59	

**Fig. 1** Photos of mesocercaria in frogs observed by microscope or stereomicroscope (**a, b, c, d**: *A. alata*; **e, f**: Strigeidae). **a** alive (obtained by Baermann modified); **b** alive encapsulated (after dissection); **c, d** fixed mesocercaria; **e** Strigeida in muscle of an adult frog; **f** fixed mesocercaria



**Fig. 2** Number of tadpoles and frogs with or without *A. alata* among water frogs and brown frogs





**Table 2** Distribution of *A. alata* mesocercaria on two water frogs

Body parts	Froglet		Sub-adult frog	
	Number of mesocercaria	Infestation proportion (%)	Number of mesocercaria	Infestation proportion (%)
Head	79	48	75	28
Periorbital	42	26	10	6
Visceral cavity	16	10	70	26
Vertebral body	20	12	66	24
Hind limb	7	4	50	18
Forelimb	0	0	0	0
Total	164		314	

## Discussion

Water frogs and brown frogs *sensu lato* are the two most widespread amphibian groups in Europe (Berger 1988; Graf and Polls Pelaz 1989; Gasc et al. 1997; Pagano et al. 2001; Duguet and Melki 2003). This study assessed their potential role as intermediate hosts for the development from furcocercariae (emitted by aquatic planorbid snails) to mesocercariae of *A. alata* on the Der-Chantecoq area where this parasite had already been identified in snails, *Planorbis planorbis* and *Anisus vortex* (Portier et al. 2012). Both water frogs and brown frogs are receptive to *A. alata* mesocercarial infection: larval and adult amphibians of both groups were found harboring mesocercariae with parasite burdens which could reach several hundred parasites per individual. These two groups had already shown to be suitable hosts for *A. alata* mesocercarial infection (Gastaldi 1854; Andreas 2006; Lukijanov et al. 2008). Other amphibians have also been shown to harbor *A. alata* mesocercaria, including newts (*Triturus* sp.) and toads (*Bufo bufo*, *Bufo calamita*, and *Bufo viridis*) (Shimalov and Shimalov 2001; Shimalov et al. 2001a), with high prevalences and parasite burdens for all three toad species (up to 1,600 mesocercaria within an individual).

During this study, we observed that the morphological identification of *Alaria*'s mesocercariae should be realized in frogs with caution as suggested by Pearson (1959), since Amphibians are known to be the hosts of other trematode *Strigea* (Dubois 1968). Indeed, we found mesocercariae belonging to another group, the *Strigeidae*, which differ from *Alaria* mesocercariae essentially by the number of glandular cells. These observations corroborate the one of Shimalov (2002) who reported larvae of *Strigea falconis*, *Strigea sphaerula*, and *Strigea striges*, parasites of avifauna, in water frogs, and *Rana arvalis*. Hence, in contrast with wild boars, it seems important to be careful with amphibian's parasites, observing all morphological features, and in complement, proceeding to molecular analysis on some mesocercariae to

confirm the identification, especially in areas where avifauna is widespread and diverse like the Der-Chantecoq.

It is interesting to note that in specific conditions, most amphibians, especially tadpoles, can feed on tadpoles from other species (predatory behavior) (*Pelodytes punctatus*, *Pelobates cultripipes*), or even on smaller tadpoles of their own species (cannibalistic behavior) (brown frogs, water frogs, salamander, newts) (Crump 1983; Pfennig and Collins 1993; Petranka and Thomas 1995; Kwet 1996; Zahn 1997; Griffiths 1997; Miaud and Muratet 2004; Balint et al. 2008). Amphibian infection by *A. alata* could occur both through infection by furcocercariae but also consumption of other infected amphibians, the first one being the principal way. Hence, amphibians could act both as intermediate and paratenic hosts for a new amphibian.

Some of these mesocercaria were observed encapsulated within the amphibian tissues (for both groups). Such encapsulation corroborates previous studies which already reported it in American species: *Alaria marciana*, *Alaria mustelae*, and *Alaria arisaemoides* (Hofer and Johnson 1970).

Adult brown frogs are the first group of amphibians to start their reproduction, between mid-January and mid-April depending on the climate in France (Augert and Joly 1993; Gasc et al. 1997; Miaud et al. 1999; Lesbarrères and Lodé 2002). This reproduction phase is very short: around 2 weeks (Wells 1977; Duguet and Melki 2003). During this reproduction period, qualified as “explosive,” adults spawn in pond water, and eggs hatch into free swimming tadpoles within approximately 3 weeks (Haapanen 1982; Elmberg 1991; Duguet and Melki 2003). After copulation, females leave the pond to find smaller male-free ponds—the summer habitat—to rest and live until the hibernation, whereas males stay around the reproductive pond during all reproduction period to copulate with the most females as possible, before reaching a summer pond. Tadpoles are exclusively aquatic until their metamorphosis, which takes between 2 and 4 months depending on temperatures (Gasc et al. 1997; Duguet and Melki 2003). After metamorphosis, froglets leave the reproductive

pond in June–July, to reach summer ponds and then hibernation habitat around September–October with the other adults. Water frogs present a similar pattern but their reproduction start later and lasts longer, between April and June (Duguet and Melki 2003). Hence, the larval development of tadpoles is shorter, around 1 and 2 months, since temperatures are higher (Berger 1973; Hotz et al. 1999). The emissions of *A. alata* cercaria by snails occur therefore during this period which closely matches the periods of high amphibian densities, with both larval and adult amphibians of both groups in the ponds.

Although both amphibian groups are susceptible to *A. alata* infection, this study shows that for the Der-Chantecoq area, prevalence of *A. alata* was higher within brown frogs than in water frogs, whichever the developmental stage. This result coincides with anterior works (Andreas 2006) and can be explained by several hypothesis: (i) a different receptivity of amphibians for this parasite according to their species, due to physiological properties related to skins' permeability or attractivity; (ii) divergent reproductive periods, which are earlier in the season for brown frogs (Gasc et al. 1997; Duguet and Melki 2003), might correspond to the period where emission of cercaria from snails is the greatest and where the abundance of snails is at the maximum (Patrelle et al., unpublished data); (iii) behavioral differences between amphibian species: water frog tadpoles are solitary and brown frog tadpoles reveal an extremely gregarious behavior, increasing their infection probability. Hence, only few snails could be enough to infest thousands of brown frog tadpoles. Furthermore, parasite burdens were equivalent between brown frogs and water frogs. This result is more in favor of behavioral differences between groups (combination of different seasonal presence and gregarious versus solitary tadpole behaviors).

No significant differences between prevalences or parasitic burdens of *A. alata* were observed between tadpoles and frogs, showing that mesocercaria do not accumulate through age within the amphibian hosts. This leads to several hypothesis: (i) only tadpoles are susceptible to mesocercarial infection but mesocercaria can survive a long time within the adult frog without affecting its survival; (ii) both stages are susceptible to infection but mesocercaria do not survive a long time within the host or affect its survival.

Some authors have reported failure to infect adult frogs with American *Alaria* species after using the same material and methods which were successful for tadpole infection (Johnson 1968). In addition, it could be possible that the mesocercarial encapsulation in the amphibian hosts serves a survival purpose, enabling mesocercaria to persist within the host for several months or maybe years. Even if lifespan of *A. alata* mesocercaria is nowadays unknown, these aspects of mesocercarial biology lead us to think that larval stages are the only susceptible stage for infection by furcocercaria and that mesocercaria will persist for a long time within adult frogs.

Another observation of this study suggests that high parasite burdens might have an impact on host survival: the localization of the mesocercaria within the host. The highest densities of *A. alata* mesocercaria were observed in the tissues around the eyes (up to a third of total burden). This observation is consistent with previous studies on *Alaria americana* (Hofer and Johnson 1970). This result lead us to suggest that such high parasite densities around the eyes might have an impact on the frog or tadpole's vision, and thus on its survival since its ability to avoid predator or to apprehend its environment could be dramatically reduced. Impacting amphibian visual abilities, *A. alata* might increase predation probabilities and facilitate its transmission to the following host (paratenic or definitive hosts). This could also have had an impact on sampling, as tadpoles were caught in nets (visual abilities were useless) and frogs were caught by hand and nets (considerable importance of visual abilities for escaping), introducing a bias toward highly parasitized adults.

Amphibians collected in Argentolle had a higher prevalence for *A. alata* mesocercaria but a lower parasitic burden than those from Coulon. These differences might be due to differential densities of positive snails harboring *A. alata* cercaria, unequal emission stimulations of snails, and/or the difference of water flow.

## Conclusion

During this study, we observed that furcocercarial emission by snails closely matched the periods of high amphibian densities on the studied areas, focused not on high amphibian densities in general, but on high tadpole densities. Even if the two groups of frogs are suitable for the parasite, the brown frogs are more often parasitized by *A. alata* than the water frogs. The observation that no increase in prevalence and parasite burdens through amphibian age strongly suggests that tadpoles are the preferential stage susceptible to infection by *A. alata* furcocercaria—as has been observed during experimental infection studies on American *Alaria* species. We suggest that mesocercaria could survive for a long time within adult frogs but also that high mesocercarial densities might have an impact on the host survival: the high parasite densities observed around the eyes of adult frogs certainly have an impact on the hosts' vision. Hence, understanding the life cycle of this kind of parasites is essential for zoonotic risk factors identification. In the future, it would therefore be worthwhile extending our research on these intermediate hosts to more amphibian species, and to other regions, especially in countries where mesocercariae have been detected in wild boar.

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