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***Alaria* mesocercariae in the tails of red-sided garter snakes: Evidence for parasite-mediated caudectomy**

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Abstract

Trematodes of the genus *Alaria* develop into an arrested stage, known as mesocercariae, within their amphibian second intermediate host. The mesocercariae are frequently transmitted to a non-obligate paratenic host before reaching a definitive host where further development and reproduction can occur. Snakes are common paratenic hosts for *Alaria* spp. with the mesocercariae often aggregating in the host's tail. In the current study, we used morphological examination and molecular analyses based on partial sequences of nuclear large ribosomal subunit gene and mitochondrial cytochrome C oxidase subunit 1 gene to identify larvae in the tails of red-sided garter snakes (*Thamnophis sirtalis parietalis*) as mesocercariae of *Alaria marcianae*, *Alaria mustelae*, and *Alaria* sp. as well as metacercariae of Diplostomidae sp. of unknown generic affiliation. We assessed infection prevalence, absolute and relative intensity, and associated pathological changes in these snakes. Infection prevalence was 100% for both male and female snakes. Infection intensity ranged from 11 to more than 2,000 mesocercariae per snake tail, but did not differ between the sexes. Gross pathological changes included tail swelling while histopathological changes included mild inflammation and the presence of mucus-filled pseudocysts surrounding mesocercariae, as well as the compression and degeneration of muscle

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fibers. Our results indicate that mesocercariae can lead to extensive muscle damage and loss in both sexes which likely increases the fragility of the tail making it more prone to breakage. As tail loss in garter snakes can affect both survival and reproduction, infection by *Alaria* mesocercariae clearly has serious fitness implications for these snakes.

Keywords

trematodes; *Alaria*; snakes; *Thamnophis*; histopathology; molecular identification

INTRODUCTION

Alaria is a genus of digenetic trematodes with a life cycle that is unusual among trematodes in that it includes a mesocercarial larval stage that frequently passes through one or more paratenic hosts (Bosma 1934; Möhl et al. 2009). As with most trematodes, eggs from adult *Alaria* spp. passed from the digestive tract of the definitive host hatch into miracidia which infect aquatic snails as first intermediate hosts (Bosma 1934; Pearson 1956; Möhl et al. 2009). Cercariae emerging from a snail next enter a frog where, rather than developing into encysted metacercariae as is typical of trematodes in their second intermediate hosts, they develop into mesocercariae (Bosma 1934; Pearson 1956; Möhl et al. 2009). The complex life cycle of *Alaria* spp. was first described in detail by Bosma (1934) who also first gave the name mesocercaria to the non-reproductive stage that does not undergo further development into the metacercarial and adult stages until ingested by a suitable definitive host, a mammalian carnivore such as a mustelid or canid (Möhl et al. 2009). Frequently, however, the infected frog is consumed by a paratenic host in which the mesocercariae will persist unchanged (Shoop 1988; Möhl et al. 2009). As a paratenic host ingests more mesocercariae, the larvae accumulate in the host's tissues increasing the number of parasites that can be transmitted to the definitive host. Thus, while the paratenic host is not physiologically necessary for the completion of the *Alaria* life cycle, it can have an ecologically important role in parasite transmission (Möhl et al. 2009).

The mesocercarial stage of *Alaria* spp. has low host specificity and a variety of paratenic hosts have been reported including snakes, opossums, raccoons, and rodents as well as game species such as wild boar and alligator (Shoop and Corkum 1981; Sharpilo and Tkach 1989; Möhl et al. 2009). Via consumption of mesocercarial infected meat, humans can also become paratenic hosts (Fernandes et al. 1976; Freeman et al. 1976; Möhl et al. 2009). Indeed, alariosis has been cited as a (re-)emerging zoonosis of particular concern in Europe (T b ran et al. 2013; Wasiluk 2013; Portier et al. 2014). Within humans, the pathological effects of *Alaria* spp. mesocercariae can be serious and at least one fatal infection involving severe pulmonary hemorrhaging has been documented (Fernandes et al. 1976; Freeman et al. 1976). Other than human case studies, relatively few investigations have specifically addressed the pathological changes associated with infection by *Alaria* spp. in paratenic hosts. This paucity of studies is particularly evident for non-mammalian species such as snakes although they appear to be some of the most commonly documented paratenic hosts.

The current study focuses on *Alaria* spp. infections in the tails of red-sided garter snakes (*Thamnophis sirtalis parietalis* (Say in James 1823)) from Manitoba, Canada. Infection by *Alaria* spp. have been frequently noted for a number of *Thamnophis* species including *T. sirtalis* (Rau and Gordon 1978; Goldberg and Bursey 2002; Jiménez-Ruiz, García-Prieto, and De León 2009). In red-sided garter snakes, necropsies have revealed the vast majority of *Alaria* spp. mesocercariae to be concentrated in the tail with very few mesocercariae found elsewhere in the body (E.J. Uhrig, pers. obs.). Additionally, several published studies indicate the propensity for mesocercariae to accumulate in the tails of snakes and observations of gross external changes consisting of a swollen tail with potential tissue necrosis have been noted (La Rue 1917; Olivier and Odlaug 1938; Odlaug 1940; Sparkman and Palacios 2009). In the field, garter snakes are often observed to have lost portions of their tails (e.g., Fig. 1e,f) and it is believed that some such injuries may be facilitated by parasite infection (La Rue 1917; Shine et al. 1999). However, no prior studies have provided detailed investigations of *Alaria* pathological changes in the tails of garter snakes despite the importance of the tail in garter snake survival and reproduction. In their study of tail loss patterns in *Thamnophis*, Willis et al. (1982) suggested that juveniles suffering tail loss were less likely to survive the stress of hibernation. With regard to reproduction, males utilize their tails in intrasexual tail wrestling competitions as they compete for access to the female's cloaca and Shine et al. (1999) found that males missing portions of their tails have reduced mating success. For female snakes, tail loss may make it more difficult for males to determine the location of the female cloaca (Pisani 1976; Perry-Richardson et al. 1990). Thus, parasite-mediated tail loss could substantially affect garter snake fitness particularly since, unlike many lizard species, these snakes are incapable of tail regeneration (Fitch 2003).

In the current study, we examined the tails of red-sided garter snakes naturally infected with *Alaria* spp. The goals of the present study with garter snakes were to 1) use molecular data to elucidate the taxonomic status of the *Alaria* spp. mesocercariae in *Thamnophis sirtalis parietalis*, 2) characterize the histopathology of *Alaria* spp. infection in garter snake tails, 3) assess the prevalence and intensity of the infections, and 4) determine whether there was evidence of intersexual variation in infection patterns or pathological changes.

MATERIALS & METHODS

Study Population

All red-sided garter snakes used in this study were wild caught individuals collected from a den site in the Interlake region of Manitoba, Canada (50°31'58"N; 97°29'71"W); the den is estimated to overwinter approximately 35,000 garter snakes (Shine et al. 2006). Collections were made by hand during the boreal spring breeding season when snakes were emerging from winter hibernation. Snakes remain aphagous throughout hibernation and the breeding season (Aleksiuk and Stewart 1971) thus any *Alaria* spp. mesocercariae contained within them were acquired during feeding in the previous summer. Following their use in a separate behavioral study, snakes (N = 16 males, 16 females) were euthanized with an injection of Beuthanasia® and their tails were immediately collected for histology and parasite counts.

Molecular Identification

As mesocercariae are morphologically similar among *Alaria* species, molecular methods of identification are particularly useful (Riehn et al. 2011). Thus, in order to make species-level identification of diplostomid larvae found in the tails of red-sided garter snakes in Manitoba, we extracted DNA and obtained sequences from larvae collected from 2 male garter snakes. The digenean larval stages found in garter snake tails could be visually separated into three groups according to their morphotypes. Two of these distinct forms were mesocercariae of *Alaria* (a larger and a smaller form) which constituted the vast majority of the larvae in the snake tails. In addition, our samples contained a very small number of diplostomid metacercariae of unclear generic affiliation that were morphologically reminiscent of the larvae traditionally referred to as *Fibricola* in the literature reporting larval stages from amphibians and reptiles (e.g., Chandler 1942; Turner 1958). We sequenced 6 larvae of *Alaria* (3 larger and 3 smaller forms) and 3 larvae of unknown diplostomids. We also sequenced diplostomid larval stages, including *Alaria* mesocercariae, from several wood frogs (*Lithobates sylvaticus* (LeConte 1825)) collected in North Dakota, a state adjoining the southern border of Manitoba. Additionally, to facilitate the identification of the larval stages, we sequenced adult specimens of *Alaria* obtained from several species of mustelid carnivores known to feed on frogs and/or garter snakes including the striped skunk (*Mephitis mephitis* Schreber 1776), American badger (*Taxidea taxus* Schreber 1777) and long-tailed weasel (*Mustela frenata* Lichtenstein 1831) (Table 1). The mustelids we examined were donated by hunters during the regular hunting season in North Dakota.

Prior to DNA extraction, the larval stages were photographed under an Olympus BX51 compound microscope for future reference. For adult specimens, we had numerous vouchers for morphological comparison. Voucher specimens of both larval and adult stages were prepared as permanent total mounts stained with alum carmine and mounted on slides in Damar gum. In all cases genomic DNA was extracted from individual specimens of either larval or adult worms following the protocol of Tkach and Pawlowski (1999). In order to increase the probability of matching our sequences from larvae to those of previously published and our own sequences of adults, we sequenced two DNA fragments. One of these DNA target regions was a 1,100 bp long fragment at the 5' end of the nuclear large ribosomal subunit RNA gene (28S) including variable domains D1–D3. In addition, for higher resolution and comparison with data previously published from Canada, we sequenced the “barcoding” region of the mitochondrial cytochrome C oxidase subunit 1 gene (cox1). Forward primer digl2 (5'-AAGCATATCACTAAGCGG -3') and reverse fragment 1500R1 (5'-GCTACTAGATGGTTCGATTAG -3') designed by V. Tkach were used for amplification and sequencing of the 28S region. Diplostomid-specific forward and reverse primers Plat-diploCOX1F (5'-CGTTTRAATTATACGGATCC-3') and Plat-diploCOX1R (5'-AGCATAGTAATMGCAGCAGC-3') designed by Moszczynska et al. (2009) were used for amplification and sequencing of the cox1 region. DNA was amplified using Polymerase Chain Reactions (PCR) on an Eppendorf EP Gradient thermal cycler using OneTaq Quick-load Mastermix from New England Biolabs (Ipswich, MA) according to manufacturer's instructions. Annealing temperature was set at 53°C for 28S amplifications and at 45°C for cox1 amplifications. PCR products were purified using an Exo-Sap IT PCR Product Clean-up kit from Affymetrix (USA), cycle-sequenced using ABI

Big-Dye™ chemistry, alcohol-precipitated, and run on an ABI Prism 3100™ automated capillary sequencer. PCR primers were used in sequencing reactions. Contiguous sequences were assembled and edited using Sequencher™ ver. 4.2 (GeneCodes Corp., Ann Arbor, MI) and submitted to the GenBank (see Table 1 for accession numbers). Sequence alignments and pairwise comparisons were done using BioEdit software, version 7.0.1 (Hall 1999).

Histopathology

Tails collected (N = 6 males, 6 females) for histology were severed approximately 1 cm anterior to the cloacal opening and injected ventrally at 1cm intervals along their length with Dietrich's fixative. The tails were then stored in 30 ml glass vials containing additional Dietrich's until histological analyses at Oregon State University. Tails were sectioned transversely and decalcified using Cal-Ex II solution. Following decalcification, tails were embedded in paraffin blocks and processed in the standard manner. Sections were placed on glass slides and stained using the standard H&E method. Slides were then evaluated via light microscopy.

Parasite Counts

Tails utilized for parasite counts (N = 10 males, 10 females) were a randomly selected subset of intact tails collected as part of a separate larger study. Tails were severed 1 cm posterior to the cloacal opening and the ventral surface was carefully slit open with scissors. Tails were stored individually in 5% formalin for later counting. Prior to counting, mesocercariae were separated from tail tissue using fine-tipped forceps under a stereomicroscope (Nikon SMZ-745T). Mesocercariae were easily recognized by their characteristic oval shape as well as their conspicuous penetration glands and acetabulum (Fig. 2). However, as the mesocercarial stage is morphologically nearly indistinguishable among species of *Alaria*, we did not attempt to differentiate *Alaria* species during parasite counts. After the tail tissue was removed, total parasite counts were conducted using a Bogorov-type plankton counting chamber under the stereomicroscope. Prevalence (percentage of individuals infected) and intensity of infection (number of parasites per infected host) were determined (Bush et al. 1997). In order to account for variation in tail length, parasite density (number of mesocercariae per cm of tail) was also calculated.

Statistics

As the non-normality of tail length data could not be corrected by statistical transformations, tail lengths were compared between sexes using Mann-Whitney Rank Sum tests. Infection intensity data passed normality and equal variance tests (Shapiro-Wilk and Brown-Forsythe tests, respectively, with $P > 0.100$ for all tests), thus male and female infection intensities were compared using Student's *t*-tests. All statistical tests were conducted in the SigmaPlot software package (version 12.5; Systat Software, Inc.).

RESULTS

Molecular Identification

The target fragment of the *cox1* gene was 470 base pairs long in all sequenced species. The sequenced fragment of the 28S gene was 1,100 base pairs long in all sequenced species. As

we were unable to produce full length 28S sequences from all specimens, the 28S alignment was trimmed to the length of the shortest sequence (975 base pairs). BLAST searches in the GenBank and alignment of newly obtained sequences of larval and adult forms demonstrated that the community of diplostomid larval stages in the tails of the red-sided garter snakes we examined included 4 species of diplostomid larvae, specifically *Alaria mustelae* Bosma 1931 (2 sequenced smaller mesocercariae), *A. marciana* (La Rue 1917) (1 sequenced smaller mesocercaria), *Alaria* sp. (3 sequenced larger mesocercariae), and Diplostomidae sp. (3 sequenced metacercariae). Our 28S sequences of *A. mustelae* from the garter snakes were identical to the 28S sequences of this species published by Pulis et al. (2011) as well as to our new sequences of adult specimens from the spotted skunk, American badger, long-tailed weasel, and to mesocercariae from the wood frog, all from North Dakota. Likewise, cox1 sequences of *A. mustelae* matched the sequences of adults from the same species of definitive hosts collected in North Dakota as well as sequences of *A. mustelae* from Canada published by Locke et al. (2011). Sequences of *A. marciana* matched the sequences of adult specimens of this species obtained from American badger in North Dakota. It should be mentioned that multiple specimens of *A. marciana* collected from the badger were morphologically fully formed adults, but none of them were ovigerous which is likely a result of accidental infection of a non-specific host since normal definitive hosts of this *Alaria* species are canid carnivores. We were unable to identify the distinctly larger form of *Alaria* mesocercariae found in garter snake tails to species level because it did not match sequences of any identified *Alaria* species available in the GenBank or our collection, although it did match a sequence of a mesocercaria from a leopard frog (*Lithobates pipiens* (Schreber 1782)) in Manitoba which was published as *Alaria* sp. 1 by Locke et al. (2011). Finally, sequences of the three metacercariae of Diplostomidae gen. sp. did not match any identified adult. Although the cox1 sequences of the metacercariae matched the sequence of larvae we obtained from two wood frogs in North Dakota as well as the sequence of metacercaria published as *Fibricola* sp. by Locke et al. (2011) from the northern leopard frog (*Lithobates pipiens*) in Manitoba, they do not belong to *Fibricola* according to unpublished data V. Tkach and collaborators obtained from adult specimens of *Fibricola*.

No intraspecific variability was observed among 28S sequences of any of the 4 species identified from the tails of garter snakes (Table 2). Low levels of intraspecific variability ranging from 0 to 2.3 percent were observed among sequences of the much more variable cox1 gene (Table 3). The differences were minimal between Manitoba and North Dakota sites and more pronounced when compared to the collecting sites in Quebec studied by Locke et al. (2011).

We counted the proportions of different diplostomid larvae in samples collected from 2 garter snake tails. Unfortunately, morphological differentiation of fixed larvae of *A. marciana* and *A. mustelae* (both smaller forms) is difficult and not possible without molecular identification or mounting all specimens and measuring morphometric characters which was not feasible considering the large numbers of larvae. Therefore, we counted these two species as a pool. One of the samples had 1228 larvae, of which 898 (73.1%) were represented by the pooled small forms, 321 (26.2%) were larger *Alaria* sp. and only 9

(0.7%) were Diplostomidae gen. sp. The second sample had 326 larvae of which 108 (33.1%) were represented by the pooled small forms, 198 (60.7%) were larger *Alaria* sp. and 20 (6.1%) were Diplostomidae gen. sp.

Gross Pathology

Mean tail length of snakes used in this study was 13.1 cm for males ($N = 16$) and 14.0 cm for females ($N = 16$). Although absolute tail length did not differ between the sexes (Mann-Whitney Rank Sum, $U = 95.5$, $P = 0.227$), relative to snout-vent length, males had significantly longer tails than females (Mann-Whitney Rank Sum, $U = 69.0$, $P = 0.027$). While some tails had no obvious external changes (Fig. 1a, b), others had visibly distended scales on their ventral surface giving the tail a swollen, ‘puffy’ appearance which was usually more pronounced distally (Fig. 1c, d). Tails used for histology included specimens that appeared fully intact as well as some, termed ‘stub tails’, which were clearly missing a distal portion of their length (Fig. 1e, f). The two most extreme stub tails, one male and one female, appeared to be missing approximately 30–50% of their length (based on typical mean tail lengths for snakes in this population; Uhrig, pers. obs.). As missing tail portions would have contained parasites as well, only intact tails were utilized for parasite counts.

Histopathology

Alaria spp. mesocercariae were present in all twelve tails examined histologically even those that did not show external morphological changes. The mesocercariae had morphology in accordance with that described by Möhl et al. (2009). General features included an oral sucker, acetabulum, penetration glands, ceca, and a tegument covered with fine spines (Fig. 2 and 3). All infections appeared to begin posterior to the cloaca with a tendency for increasing numbers of mesocercariae toward the caudal end of the tail. Histologically, the infection did not appear to differ in any consistent manner between male and female snakes and the following pathological descriptions are applicable to both sexes.

Mesocercariae were present between fascial planes and were frequently surrounded by lamellar accumulations of pale, flocculent, basophilic mucinous material (Fig. 4a,b). These accumulations were occasionally surrounded by trabeculae or capsules of loosely organized fibrous connective tissue (granulation tissue) that was frequently invaded and expanded by mats of epithelioid macrophages, with fewer heterophils and occasional eosinophils (Fig. 4c,d). These walled-off compartments of mucous and inflammatory cells could be described as pseudocysts (Fig. 4a–c) since they are circular and encapsulated, but are not lined by a functional epithelium.

In most mild cases, mesocercariae tended to be confined to the fascia just ventral to the vertebral bodies that surrounded the caudal aorta and vena cava (Fig. 5a). In more severe cases, parasites appeared to spread ventrally and laterally into the rest of the fascia, frequently ending up in a subcutaneous position with no muscle between the parasite and the deep dermis (Fig. 5b–d).

Inflammation was not present in all animals and, in most cases, the only visible tissue damage associated with the parasites was the mass effect of the trematodes themselves,

which would occasionally compress adjacent muscle bundles, producing contraction bands, loss of cross-striations, fragmented cytoplasm, and occasionally atrophy (Fig. 6a,c). In many cases, there was obvious compression of adjacent muscles without any evidence of myodegeneration (Fig. 6b). The majority of cases examined were characterized only by mild inflammation. In severe cases, however, tail cross sections often contained more parasites than actual host tissue: muscle bellies were completely obliterated and replaced by thin strands of fibroplasia, atrophied muscle, and inflammatory cells delineating large pseudocysts (Fig. 5c–d, 6d).

Parasite Counts

Prevalence of *Alaria* spp. mesocercariae was 100% in both male and female snakes. Overall infection intensity ranged from 37 to 2286 mesocercariae in males and 11 to 2316 in females with mean values ($\pm$ SE) of 966.4 ($\pm$ 273.8) and 1486.5 ($\pm$ 214.9) mesocercariae respectively for males and females. Mean infection density ($\pm$ SE) was 66.3 ($\pm$ 18.8) mesocercariae per cm of tail for males and 98.7 ($\pm$ 13.8) mesocercariae per cm of tail for females. Intersexual differences in infection intensity and density were not significant (Student's *t*-tests, *P* > 0.100 for both).

DISCUSSION

Comparison of 28S and *cox1* sequences obtained from larval and adult digeneans collected in adjacent geographical regions allowed us to make species level identification for 2 out of 3 forms of *Alaria* mesocercariae infecting the tail tissues of red-sided garter snakes from our study population in Manitoba. In addition to finding these *Alaria* species in definitive hosts (mustelid carnivores), we have also found some of them in amphibian second intermediate hosts, which are common prey of garter snakes. Although we could not reliably differentiate mesocercariae of *A. marciana* and *A. mustelae* when counting large samples, it is obvious that *Alaria* mesocercariae overwhelmingly dominate the community of digenean larvae in tail tissues of garter snakes. As metacercariae of Diplostomidae gen. sp. constituted only 0.7–6.1% of the larvae found in the snake tails, their importance in the observed pathological changes is minimal to negligible. Therefore, in the subsequent discussion we consider only *Alaria* mesocercariae.

The results of our pathological examinations indicate that the presence of *Alaria* spp. mesocercariae within the tails of garter snakes has notable pathological consequences with the potential to impact host fitness. Although there was relatively little inflammation present in most samples, the mesocercariae appeared to have a compressive effect causing atrophy of muscle fibers with the more severe cases having few or no intact muscle bellies visible. In these cases, the ventral interior of the tail had essentially become a pocket of parasites and loose fibrous connective tissue. Such extensive loss of muscle would leave only intervertebral ligaments and skin attaching the tail to the rest of the body. Weakening the tail in this manner would make it more prone to breakage. Our results, therefore, support the hypothesis of parasite-mediated tail loss as suggested by La Rue (1917) and Shine et al. (1999). Our results of increasing mesocercariae in the caudal direction may also help explain observations made by Fitch (2003) in a study of tail breakage in *Thamnophis sirtalis*.

(Linnaeus 1758). Although his study made no mention of parasites, Fitch (2003) observed that some regions of the tail seemed more fragile and noted nonrandom breakage patterns with all breaks occurring caudal to the 17th percentile of tail length and many females having breaks at the 20–29th percentiles. Such regional fragility could correspond to areas of greater mesocercariae aggregation.

While garter snakes are known to autotomize their tails as a defense strategy (Cooper and Alfieri 1993; Fitch 2003; Placyk and Burghardt 2005), tail loss is not so readily employed as it is by many lizard species. Cooper and Alfieri (1993) report tail autotomy only when a snake was grasped firmly by the tail and a characteristic body rolling behavior occurred to facilitate the break. For garter snakes, tail loss is permanent and has negative fitness consequences via reduced survival and decreased mating success (Willis et al. 1982; Shine et al. 1999), thus, it seems likely that autotomy would only occur as a last resort escape strategy. With *Alaria* infection damaging and weakening the tail, breakage may occur in predator encounters where it otherwise would not be necessary. Additionally, if fragility is high enough, merely moving through the environment could result in tail breakage. Indeed, one of us (E.J. Uhrig) has observed a captive snake lose its tail while housed in a laboratory aquarium free from predators.

The phenomenon of parasites aggregating in an autotomizable appendage has been documented in other species such as Canarian lizards (*Gallotia galloti* (Oudart 1839)) and spiny sand crabs (*Blepharipoda occidentalis* Randall 1840) (Matuschka and Bannert 1987; Lafferty 1999). Lafferty (1999) suggested that such aggregation would be beneficial to trophically transmitted parasites with parasites evolving to influence the host's tendency to lose the appendage in order to facilitate trophic transmission. Indeed, this seems a plausible ultimate explanation for the tendency of *Alaria* to aggregate in the snake tails. That is, by increasing the chance of tail breakage during a predator encounter, mesocercariae increase the likelihood they will be transmitted to a subsequent host where they may be able to continue their life cycle. The propensity for predation attempts to result in tail loss was highlighted in a study by Placyk and Burghardt (2005) who found tail loss to be positively correlated with predator diversity for *Thamnophis sirtalis sirtalis* in Michigan. For *Alaria marcianae* and *A. mustelae*, which have previously been noted in coinfections (Hofer and Johnson 1970; Johnson 1979), definitive hosts include cats, raccoons, foxes, skunks, mink, and badgers all of which are potential snake predators found within the garter snakes' range in Manitoba (Bosma 1934; Johnson 1968; Johnson 1979).

Although helminth infections are often associated with inflammation and eosinophilia (Behm and Ovington 2000; Klion and Nutman 2004; Anthony et al. 2007; Foster and Elsheika 2012), such a response was relatively lacking around the mesocercariae in the snakes' tails. However, this finding is in accordance with other studies of *Alaria* spp. (e.g., Fernandes et al. 1976; Shoop and Corkum 1984) which similarly noted a lack of inflammation. Of related interest is our observation of 'pseudocysts' appearing as mucus-filled compartments often containing multiple mesocercariae. While the nature of our study did not allow us to definitively determine whether the mucus was host or parasite derived, a study by Hofer and Johnson (1970) of *Alaria mustelae* mesocercarial capsules found no evidence of mucus producing cells in the parasite and concluded, therefore, that the mucus is

most likely produced by the host. Regardless of the mucus' origin, however, it is probable that the pseudocystic capsules around the mesocercariae contribute to the reduced inflammatory response. Similar immune-shielding effects of mucus production and encapsulation have been noted in other studies of parasitic infections (e.g., Riffkin et al. 1996; Theodoropoulos et al. 2001; Fernandes et al. 2012).

Although intersexual variation in various aspects of parasitic infections has been reported for a number of other species (Poulin 1996; Zuk and McKean 1996), we found no significant intersexual variation in our study. Nevertheless, with our focus on the infection in the tail, we cannot definitively rule out the possibility that intersexual variation may exist if all mesocercariae within each snake's body were considered. However, such variation seems unlikely given that typically very few mesocercariae are found elsewhere besides the tail (E.J. Uhrig, pers. obs.). Our finding of 100% prevalence for both sexes is not surprising given that *Alaria* is acquired trophically and frogs, the parasite's second intermediate host, are a major component of the garter snakes' diet (Gregory and Stewart 1975). While diet composition does not differ between males and females (Gregory and Stewart 1975), we might have expected to see significant variation in infection density because female snakes, being larger in size than males, may consume more or larger frogs and so ingest more mesocercariae. Additionally, despite their overall larger size, the tails of females are shorter relative to body length than are male tails (Shine et al. 1999). Thus, we could reasonably expect more mesocercariae per cm of tail for females. However, despite a slight trend for females to have higher infection density than males, intersexual differences were not significant. Given that individual frogs can harbor thousands of mesocercariae (Fernandes et al. 1976) and a snake will eat many frogs over its lifetime, clearly not all mesocercariae consumed become permanently established in the tail. Thus, snakes may have some degree of resistance to the parasite and it is conceivable that such resistance could vary between the sexes (Zuk 2009; McClelland and Smith 2011). If females have greater resistance than males, as has been shown in a number of other species (Klein 2000), then, despite ingesting greater numbers of parasites, fewer mesocercariae may be able to establish in female tails leading to the lack of intersexual variation in infection intensity that we observed. This explanation, however, is somewhat speculative and clearly the topic of parasite resistance in garter snakes is one that requires further investigation.

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Fig. 1.

Tail morphology of male (images on the left) and female (images on the right) red-sided garter snakes (*Thamnophis sirtalis parietalis*). Images A and B depict the ventral surfaces of tails with no obvious external changes. Images C and D exemplify a “puffy” tail morphology where the ventral surface is swollen with distended scales particularly near the distal end of the tail. Images E and F depict a “stub” tail morphology where a portion, possibly up to 50%, of the tail is missing. Photo credit: E.J. Bentz

Fig. 2.
Wet mount of *Alaria* spp. mesocercaria from the tail of a red-sided garter snake
(*Thamnophis sirtalis parietalis*)

Fig. 3.

Two different views of *Alaria* spp. mesocercariae from the tails of red-sided garter snakes (*Thamnophis sirtalis parietalis*). Key: A. acetabulum. B. gland cells. C. cecum. D. duct of penetration gland. E. esophagus. F. penetration gland. G. oral opening with oral sucker

Fig. 4.

Patterns of host response to mesocercariae in the tails of red-sided garter snakes (*Thamnophis sirtalis parietalis*). A. Single mesocercaria surrounded by lamellar flocculent, basophilic mucinous material (star) encapsulated in a pseudocyst formed by a layer of epithelioid macrophages and fibroblasts (arrow). B. Multifocal accretions of lamellar mucinous material occasionally mineralized at the edges. C. A pair of mesocercariae in a pseudocyst lacking mucinous material but still surrounded by a thin layer of epithelioid macrophages and fibrous connective tissue (arrow). The surrounding epimysium and perimysium of muscle bundles along with intermuscular fascia is expanded by edema fluid, heterophils, macrophages, and eosinophils. While multiple mesocercariae are observed in close association and occasionally appear to adhere to each other via acetabula, whether this is a feature of the life cycle or a simple sequela of high density within a small space is unclear. D. Multiple mesocercariae not encapsulated by pseudocysts but separated by broad trabeculae of loose fibrous connective tissue (arrow). These mesocercariae have effaced the vast majority of host muscle in this region and are present just under the dermis (star)

Fig. 5.

Potentially progressive patterns of tissue invasion in the tails of red-sided garter snakes (*Thamnophis sirtalis parietalis*). A. Mesocercariae in a relatively mild infection located just ventral to the vertebrae and surrounding large tail vessels expanding the fascia with mucus. B. Mesocercariae in a more severe infection extending through the midline fascia (arrow). C. Mesocercariae in a progressively more severe infection extending from the midline fascia ventral to the spine and spreading subcutaneously along the ventrum (arrow). D. Severe infection from a relatively anterior portion of the affected tail in which mesocercariae have massively expanded the dorsolateral subcutis (arrow)

Fig. 6.

Host tissue responses to infection in the tails of red-sided garter snakes (*Thamnophis sirtalis parietalis*). A. A combination of acute and chronic changes. Muscle bundles are separated by connective tissue expanded by edema fluid, mild fibrosis, and a mild inflammatory infiltrate composed of eosinophils, macrophages, and fewer heterophils (stars). Multifocal muscle fibers have fragmented or vacuolated cytoplasm, an acute degenerative change (arrow). B. Mesocercariae can invade fascial planes between muscle bellies with minimal inflammation and muscle damage. C. Bands of fibrous connective tissue and inflammatory cells associated with mesocercariae can surround and entrap degenerate myofibers (arrow). D. Mesocercaria surrounded by abundant inflammation composed of loose, fibroblast-rich trabeculae infused with macrophages and eosinophils as well as rafts of free epithelioid macrophages, heterophils, and eosinophils (arrow)

Table 1

Digeneans used for molecular identification, their hosts, geographical origin of material and GenBank accession numbers for corresponding sequences. Sequences previously available in the GenBank are marked by “**”.

Digenean taxa	Stage	Host species	Geographic origin	GenBank numbers.	
				28S	cox1
<i>Alaria mustelae</i>	Mesocercaria	Red-sided garter snake, <i>Thamnophis sirtalis parietalis</i>	Interlake region, Manitoba, Canada	KT254014	KT254032
<i>Alaria mustelae</i>	Mesocercaria	Wood frog, <i>Lithobates sylvaticus</i>	Nelson County, North Dakota	KT254015	-
<i>Alaria mustelae</i>	Mesocercaria	Wood frog, <i>Lithobates sylvaticus</i>	Nelson County, North Dakota	KT254016	-
<i>Alaria mustelae</i>	Mesocercaria	Long-tailed weasel, <i>Mustela frenata</i>	Grand Forks County, North Dakota, USA	KT254017	KT254033
<i>Alaria mustelae</i>	Mesocercaria	American badger, <i>Taxidea taxus</i>	Nelson County, North Dakota, USA	KT254018	KT254034
<i>Alaria mustelae</i>	Mesocercaria	American badger, <i>Taxidea taxus</i>	Nelson County, North Dakota, USA	KT254019	KT254035
<i>Alaria mustelae</i>	Mesocercaria	Striped skunk, <i>Mephitis mephitis</i>	Nelson County, North Dakota, USA	KT254020	KT254036
<i>Alaria mustelae</i>	Mesocercaria	Northern Leopard frog, <i>Lithobates pipiens</i>	Quebec, Canada	-	JF769436*
<i>Alaria mustelae</i>	Mesocercaria	Striped skunk, <i>Mephitis mephitis</i>	North Dakota, USA	JF820605*	-
<i>Alaria mustelae</i>	not specified	not specified	New Hampshire, USA	-	JF904529*
<i>Alaria marcianae</i>	Mesocercaria	Red-sided garter snake, <i>Thamnophis sirtalis parietalis</i>	Interlake region, Manitoba, Canada	-	KT254038
<i>Alaria marcianae</i>	Mesocercaria	Red-sided garter snake, <i>Thamnophis sirtalis parietalis</i>	Interlake region, Manitoba, Canada	KT254021	KT254039
<i>Alaria marcianae</i>	Mesocercaria	American badger, <i>Taxidea taxus</i>	Nelson County, North Dakota, USA	KT254022	KT254037
<i>Alaria</i> sp.	Mesocercaria	Red-sided garter snake, <i>Thamnophis sirtalis parietalis</i>	Interlake region, Manitoba, Canada	KT254023	KT254040
<i>Alaria</i> sp.	Mesocercaria	Red-sided garter snake, <i>Thamnophis sirtalis parietalis</i>	Interlake region, Manitoba, Canada	KT254024	KT254041
<i>Alaria</i> sp.	Mesocercaria	Red-sided garter snake, <i>Thamnophis sirtalis parietalis</i>	Interlake region, Manitoba, Canada	KT254025	KT254042
<i>Alaria</i> sp.	Mesocercaria	Wood frog, <i>Lithobates sylvaticus</i>	Nelson County, North Dakota	KT254026	-
<i>Alaria</i> sp. 1	Mesocercaria	Northern Leopard frog, <i>Lithobates pipiens</i>	Quebec, Canada	-	JF769439*
<i>Diplostomidae</i> sp.	Metacercaria	Red-sided garter snake, <i>Thamnophis sirtalis parietalis</i>	Interlake region, Manitoba, Canada	KT254027	KT254043
<i>Diplostomidae</i> sp.	Metacercaria	Red-sided garter snake, <i>Thamnophis sirtalis parietalis</i>	Interlake region, Manitoba, Canada	KT254028	KT254044
<i>Diplostomidae</i> sp.	Metacercaria	Red-sided garter snake, <i>Thamnophis sirtalis parietalis</i>	Interlake region, Manitoba, Canada	KT254029	KT254045
<i>Diplostomidae</i> sp. 1184	Metacercaria	Wood frog, <i>Lithobates sylvaticus</i>	Nelson County, North Dakota	KT254030	-
<i>Diplostomidae</i> sp. 774	Metacercaria	Wood frog, <i>Lithobates sylvaticus</i>	Nelson County, North Dakota, USA	KT254031	-
<i>Fibricola</i> sp. 1	not specified	not specified	not specified	-	JF904537*

Table 2

Pairwise base pair variability of partial 28S rRNA sequences (975 bp fragment) among studied larval stages of the Diplostomidae. Numbers of number of variable positions are provided above the diagonal and percentages of variable positions are provided below the diagonal. No intraspecific differences were detected in 28S sequences.

	<i>A. mustelae</i>	<i>A. marcianae</i>	<i>Alaria</i> sp.	<i>Diplostomidae</i> sp.
<i>A. mustelae</i>	-	9	11	63
<i>A. marcianae</i>	1.0	-	8	62
<i>Alaria</i> sp.	1.2	0.9	-	64
<i>Diplostomidae</i> sp.	6.5	6.4	6.6	-

Table 3

Pairwise base pair variability of partial cox1 sequences (470 bp fragment) among studied larval stages of the Diplostomidae. Numbers of base pair differences are provided above the diagonal, percentages of variable sites are provided below the diagonal. The cells in the diagonal represent ranges of intraspecific variability (number of variable positions/percentage of variable positions).

	<i>A. mustelae</i>	<i>A. marcianae</i>	<i>Alaria</i> sp.	<i>Diplostomidae</i> sp.
<i>A. mustelae</i>	0–11/0–2.3	48–51	58–61	59–61
<i>A. marcianae</i>	89.1–89.8	0–1/0–0.2	44–46	59–60
<i>Alaria</i> sp.	12.3–13	9.4–9.8	0–1/0–0.2	63–65
<i>Diplostomidae</i> sp.	12.5–13	12.5–12.8	13.4–13.8	0–4/0–0.9