

Variable infection of stream salamanders in the southern Appalachians by the trematode *Metagonimoides oregonensis* (family: Heterophyidae)

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Abstract Many factors contribute to parasites varying in host specificity and distribution among potential hosts. *Metagonimoides oregonensis* is a digenetic trematode that uses stream-dwelling plethodontid salamanders as second intermediate hosts in the Eastern US. We completed a field survey to identify which stream salamander species, at a regional level, are most likely to be important for transmission to raccoon definitive hosts. We surveyed six plethodontid species ($N=289$ salamanders) from 23 Appalachian headwater sites in North Carolina: *Desmognathus quadramaculatus* ($n=69$), *Eurycea wilderae* ($n=160$), *Desmognathus ocoee* ($n=31$), *Desmognathus monticola* ($n=3$), *Eurycea guttolineata* ($n=7$), and *Gyrinophilus porphyriticus* ($n=19$). We found infection in all species except *D. monticola*. Further analysis focused on comparing infection in the two most abundant species, *D. quadramaculatus* and *E. wilderae*. We found that *D. quadramaculatus* had significantly higher infection prevalence and intensity, probably due to a longer aquatic larval period and larger body sizes and thus greater cumulative exposure to the parasite.

Keywords *Desmognathus* · *Elimia* · *Eurycea* · Heterophyid · Plethodontidae

Introduction

All animals serve as hosts to a diverse array of parasites; however, parasites are rarely evenly distributed among individuals within a host population. Often, a few individuals tend to have many parasites and most individuals have very few (Shaw and Dobson 1995; Poulin 2007), leading to parasite infection intensity data from natural populations fitting a negative binomial distribution (Crofton 1971). There has been substantial debate about the factors that cause these non-normal distributions, but differential exposure, variation in host susceptibility, parasite selection of preferred hosts, and host condition may all contribute to this pattern (Crofton 1971; Poulin 2007; Morrill and Forbes 2012). For parasites that infect multiple host species within a community, the distribution of parasites among host species is also often not a normal distribution (Poulin 2005). While host physiology and behavior and parasite selectivity may contribute to differential use of potential host species, other ecological factors, such as the relative abundance of the different host species in the community, and their life histories, may also play a role (Poulin 1992; Adamson and Caira 1994).

Metagonimoides oregonensis is a heterophyid trematode associated with freshwater streams. Adult *M. oregonensis* live and sexually reproduce primarily in the small intestine of raccoons, *Procyon lotor* (Price 1931; Sawyer 1958; Schaffer et al. 1961; Harkema and Miller 1964; Bafundo et al. 1980), although infection of mink, *Mustela vison*, has also been

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reported on occasion (Miller and Harkema 1964). Eggs are shed with the raccoon feces. If they land in a stream in the southeastern US, miracidia infect the first intermediate host, *Elimia proxima* (= *Pleurocera* = *Goniobasis proxima*), a pleurocerid snail. The miracidia develop into rediae, which in turn produce cercariae. From this point, the cercariae can either encyst in the snail as metacercariae or leave the snail to infect another host; infection of a distinct second intermediate host seems to vary across the range of the parasite (Burns and Pratt 1953; Lang and Gleason 1967). In North Carolina, stream-dwelling plethodontid salamanders (e.g., *Desmognathus* spp., *Eurycea* spp.) seem to be common second intermediate hosts (Goater et al. 1987; Goater 1990; Belden et al. 2012). After making contact with the host, the cercariae penetrate the skin and encyst as metacercariae in striated muscle tissue. The life cycle is complete when infective cysts within the salamander host are consumed by a definitive host (Burns and Pratt 1953).

The southern Appalachian region is a global hotspot for stream salamander diversity, with as many as eight species routinely occurring within a single stream reach. This results in a large number of potential second intermediate host species for *M. oregonensis*; however, salamander species vary dramatically in abundance, larval period, habitat preference, and body size (Bruce 1982, 1985, 1989; Hairston 1986). Therefore, *M. oregonensis* infection parameters, such as prevalence and intensity, may vary among potential host species. In his survey of North Carolina salamanders, Rankin (1937) found unknown metacercariae in the muscle tissue of *Desmognathus fuscus* and *Desmognathus quadramaculatus*, which appear consistent with a *M. oregonensis* infection.

Burns and Pratt (1953) were the first to experimentally complete the life cycle of *M. oregonensis* by infecting several ranid frog species with cercariae, and Lang and Gleason (1967) also experimentally infected a variety of amphibians with cercariae, including *D. fuscus*, and multiple frog species. Although *M. oregonensis* appears capable of infecting frogs, frogs are far less common than salamanders in Appalachian streams. Goater et al. (1987) conducted a survey of North Carolina salamanders and found what were suspected to be *M. oregonensis* metacercariae in the musculature of *D. quadramaculatus* and *Desmognathus marmoratus*, although they did not quantify the infection intensity. In a second study, Goater (1990) also found suspected *M. oregonensis* infection in *D. quadramaculatus* and *D. marmoratus*, but did not find any infection in *Desmognathus ochrophaeus* or *Desmognathus monticola* possibly due to a lack of ecological overlap of the more terrestrial *D. ochrophaeus* and *D. monticola* with the aquatic snails and cercariae. More recently, Belden et al. (2012) found infection levels ranging from 53 to 687 metacercariae in larval *D. quadramaculatus* (13–26 mm SVL) collected in North Carolina.

We were interested in comparing the relative prevalence of *M. oregonensis* among salamander species in the Appalachian region. Our goal was to examine multiple salamander species common to the southern Appalachian region to determine which species are naturally infected, and whether there are differences in prevalence or intensity of infection among the most abundant host species that could influence potential transmission to raccoon definitive hosts. Stream salamander species in this region vary greatly in their abundance, body sizes, habitat preferences, and larval period (Bruce 1982, 1985, 1989; Hairston 1986). For example, *D. quadramaculatus* adults tend to stay in the streams following metamorphosis where they commonly burrow into the stream banks (Petranka 1998). In contrast, *E. wilderae* become more terrestrial as adults and disperse to the forest floor (Petranka and Smith 2005). As a result of these differences in life history, *D. quadramaculatus* may be exposed at all life stages, whereas exposure of *E. wilderae* may be restricted to the relatively brief aquatic larval stage. In addition, *E. wilderae* tend to be smaller during their time in the streams, making them smaller targets for parasites. We predicted that life history, specifically the length of the aquatic larval period and accompanying differences in larval size, would play a strong role in determining infection prevalence and intensity for hosts across the region, as the more time individuals spend in a stream, the more time they have to accumulate infection.

Methods

Field collections

As part of a larger study assessing salamander occupancy probability (described in Cecala 2012; Webster et al. 2012), stream salamanders were collected from 23 different headwater streams within the upper Little Tennessee River Basin in North Carolina in 2009. Salamanders were collected under a North Carolina collecting permit (2009–268) with approval from the University of Georgia Institutional Animal Care Committee (A2009-10102). The design of this occupancy study means that our host sample sizes, both within and across streams, reflect real abundance differences in salamander species in the region. We made no attempt to increase sample sizes to specifically assess among site variation, as many of these salamander species are also considered at risk due to land use changes and climate change (Dodd and Smith 2003; Price et al. 2006). In addition, as definitive host raccoons have relatively large home ranges (Urban 1970; Gehrt and Fritzell 1997) and likely move among multiple streams within this region, we think our samples are representative of the potential pool of second intermediate hosts that would have been available to a foraging raccoon during our sampling period.

At each site, a 150-m reach was identified upstream of nearby road crossings. Within the reach 1 m² plots were created every 5 m. Each plot contained a 25 × 40 cm leaf litter bag constructed from a 1-cm² plastic mesh and filled with leaf litter from the stream banks or the nearest upstream source. After 48 h, the bags were removed from the stream, placed in a bucket, and gently agitated in water to free salamanders. The water, debris, and salamanders were then poured through a net to separate out the salamanders. After the leaf litter bags were removed, each plot was actively searched by turning cobble and dip-netting the area. Each plot was resampled over three consecutive days, and on days 1 and 2, individuals were identified and released. On day 3, individuals were collected, euthanized with an overdose of MS-222 (tricaine methanesulfonate), fixed in 10 % buffered formalin, and stored in 35 % ethanol. Identification was based primarily on Jensen et al. (2008). Specimens were transferred to 70 % ethanol prior to clearing and staining to determine *M. oregonensis* infection intensity.

Our final sample set included 289 salamanders representing six species: *D. quadramaculatus* ($n=67$ larvae, $n=2$ adults), *Eurycea wilderae* ($n=148$ larvae, $n=12$ adults), *D. monticola* ($n=3$ adults), *Desmognathus ocoee* ($n=31$ adults), *Gyrinophilus porphyriticus* ($n=19$ larvae), and *Eurycea guttolineata* ($n=7$ larvae). These sample sizes mirror natural relative abundances in this region, with *D. quadramaculatus* and *E. wilderae* as the most abundant species (Milanovich 2010). In addition, *D. quadramaculatus* and *E. wilderae* were present in 12 and 16 of the 23 streams, respectively, and they both occurred in 9 streams. Larvae were the primary age class collected because they are highly abundant during the breeding season when these salamanders were collected. We included adults that were collected in our analysis to provide an overall better representation of infection among these species.

Salamander parasite identification and estimation

M. oregonensis, as currently recognized, could be a species complex (reviewed in Belden et al. 2012). In our prior snail survey of six streams in North Carolina, *Metagonimoides* was identified as the only trematode in these stream systems that infects amphibians (Belden et al. 2012). The life cycle description of Burns and Pratt (1953) states that experimental infections of frogs with cercariae resulted in “encysted metacercariae primarily in striated muscle tissue,” and a description of metacercariae obtained from those experimental infections in Oregon suggests an average size, eyespots, and “the posterior Y-shaped excretory bladder,” which Burns and Pratt (1953) describe as “the most characteristic and conspicuous feature of the metacercaria.” That description fits with the morphology of the metacercariae we report here, although we did not examine each metacercaria counted in this study.

Taken together, all of these lines of evidence suggest we are working on what is currently recognized as *M. oregonensis*.

To assess *M. oregonensis* infection, we cleared and stained the animals using a modified version of the protocol of Hanken and Wassersug (1981) as follows. We transferred each specimen from the 70 % ethanol it was stored in to a 50-mL centrifuge tube containing 0.01 % Alcian blue cartilage stain for 24 h. Next, we transferred each specimen to EtoH:acetic acid (50:50) for 24 h, followed by 100 % ethanol for 24 h, then we soaked each specimen in tapwater for 24 h, and transferred it to 1 % trypsin in 30 % saturated sodium borate solution to clear the tissues. Specimens were left in this solution until they were limp, and blue cartilage stain was visible (24–96 h). Next, we transferred the specimens to Alcian red in 0.5 % KOH for 24 h to stain bones and trematodes within the metacercarial cysts. Specimens were then moved through two series of 24 h 0.5 % KOH rinses before a four-part series of KOH:glycerin stepdowns, from 2:1, to 1:1, to 1:2 to 100 % glycerin. For the larger, more darkly pigmented individuals, we added two to four drops of hydrogen peroxide to the 2:1 KOH:glycerin solution to help bleach the pigment. We stored specimens in 100 % glycerin. After clearing and staining, we counted all metacercariae that were visible from the ventral side bounded by the four limbs under a dissecting microscope, and photographed each specimen.

Statistical analysis

Because of limited sample sizes for four of the six species (*D. monticola*, *D. ocoee*, *G. porphyriticus*, and *E. guttolineata*), we focused our statistical comparisons only on the two most abundant species, which are also the ones most likely to be encountered by raccoon definitive hosts: *D. quadramaculatus* ($n=69$) and *E. wilderae* ($n=160$). We compared infection prevalence between these two species using Fisher’s exact test, and 95 % confidence intervals were calculated using the Wilson score interval (Zelmer 2013). To predict the number of metacercariae in an individual (infection intensity), we used a generalized linear model with a negative binomial error distribution and an identity link, with species as a categorical factor (*D. quadramaculatus* or *E. wilderae*) and snout-vent length as a continuous covariate. We chose an identity link based on biological grounds because we expect salamanders to accrue parasites at a constant rate throughout their larval periods. Comparing the AIC of an identity link model with the log link function also supported the use of the identity link. Metacercariae counts of four *D. quadramaculatus* salamanders were an order of magnitude higher than the rest and were excluded from the analysis despite their possible biological importance. No model accounted for these four points and still offered a reasonable description of the data. We analyzed the data without those points and with the caveat that very few individuals will get

very high infections, as typical of an aggregated parasite infection model.

Results

M. oregonensis infection was found in five of the six species of salamanders (in all species except *D. monticola*; Table 1). Infection prevalence was highly variable among all species and ranged from approximately 5–48 %. Infection prevalence differed between *E. wilderae* (14.4 %, CI 0.093<0.144<0.207) and *D. quadramaculatus* (47.8 %, CI 0.356<0.478<0.602; Fisher's exact test, $p<0.0001$). Individual infection intensity varied among species (Table 1; median of 4–133), and for *D. quadramaculatus* and *E. wilderae*, where we had large enough samples to look at the distribution of infection, they were aggregated (Fig. 1). Median infection intensity for *D. quadramaculatus* (133 metacercariae) was approximately 19× greater than infection intensity for *E. wilderae* (7 metacercariae; Fig. 1).

Metacercariae infection intensity increased with increasing body size (snout-vent length), which correlates with age for each of these species although *D. quadramaculatus* hatches at a larger size and has a faster growth rate. Overall infection intensity also varied between the two species (parameter estimate = -16.2 ± 3.8 , $p<0.0001$, Fig. 2), as did the rate of parasite accrual (parameter estimate = 287.4 ± 73.1 , $p<0.0001$, Fig. 2). *D. quadramaculatus* had more parasites in general and accrued more parasites relative to body size (as illustrated by the steeper slope in Fig. 2).

Discussion

M. oregonensis metacercariae were present in five of the six salamander species we collected. We did not detect any infection in *D. monticola*, which is likely the result of a small sample size ($n=3$); however, Goater et al. (1987) also did not see infection in this species, and it could be because their preference for stream banks and small seepages may limit their exposure to cercariae. It seems improbable that

M. oregonensis cannot infect *D. monticola* given that we detected the parasite in other *Desmognathus* spp., as well as distantly related confamilial species. While our results do indicate that *M. oregonensis* can infect a variety of stream salamander species, our data suggest that *D. quadramaculatus* might be particularly important in transmission dynamics of this parasite given the higher prevalence and intensity of infection found in that species, and the fact that they are one of the most abundant salamander species in these streams. This is consistent with the findings of Belden et al. (2012), who also hypothesized that *D. quadramaculatus* may be an important second intermediate host for this parasite, as they found very high infection intensities (up to 687 metacercariae) in *D. quadramaculatus* larvae.

Several factors might account for the variation among species in infection prevalence and intensity. Life history traits, and in particular the length of the larval period and body size, as well as time spent in the stream as an adult following metamorphosis, could influence the intensity of infection. All of the salamander species we examined have an aquatic larval period, but it varies in duration, with *D. quadramaculatus* having the longest larval period, lasting 36–48 months (Bruce 1985, 1988a). In contrast, *E. wilderae* has a larval period of 12–24 months (Bruce 1988b). Longer larval periods may result in a higher intensity of infection, as we saw in our comparison of *D. quadramaculatus* and *E. wilderae* because of increased exposure time to snails that are shedding parasites. *D. ocoee* (9–10 months), *D. monticola* (9–10 months), and *E. guttolineata* (5–16 months) all have much shorter larval periods (Bruce 1982, 1989). The one *G. porphyriticus* larva that was infected was the only other larva to have an infection intensity (133 metacercariae) comparable to the highly infected *D. quadramaculatus*. And similar to *D. quadramaculatus*, *G. porphyriticus* has a 36–60-month larval period (Bruce 1980).

The low numbers of *D. monticola*, *D. ocoee*, *E. guttolineata*, and *G. porphyriticus* larvae in our samples resulted in our having less ability to definitively assess infection parameters in those species. However, these sample sizes also indicate that these species are less abundant in these streams and so are likely relatively rare targets for *M. oregonensis* in this region,

Table 1 Prevalence and range of infection intensity of *M. oregonensis* in the six salamander species collected, in decreasing order of sample size

Host species	Infected/total (prevalence)	Prevalence 95 % confidence interval	Range intensity (median)
<i>Eurycea wilderae</i>	23/160 (14.4)	0.093<0.144<0.207	1–59 (7)
<i>Desmognathus quadramaculatus</i>	33/69 (47.8)	0.356<0.478<0.602	1–3321 (133)
<i>Desmognathus ocoee</i>	7/31 (22.5)	0.114<0.225<0.398	1–21 (4)
<i>Gyrinophilus porphyriticus</i>	1/19 (5.2)	0.009<0.052<0.246	136 (–)
<i>Eurycea guttolineata</i>	2/7 (28.6)	0.082<0.285<0.641	8–13 (10.5)
<i>Desmognathus monticola</i>	0/3 (0)	NA	– (–)

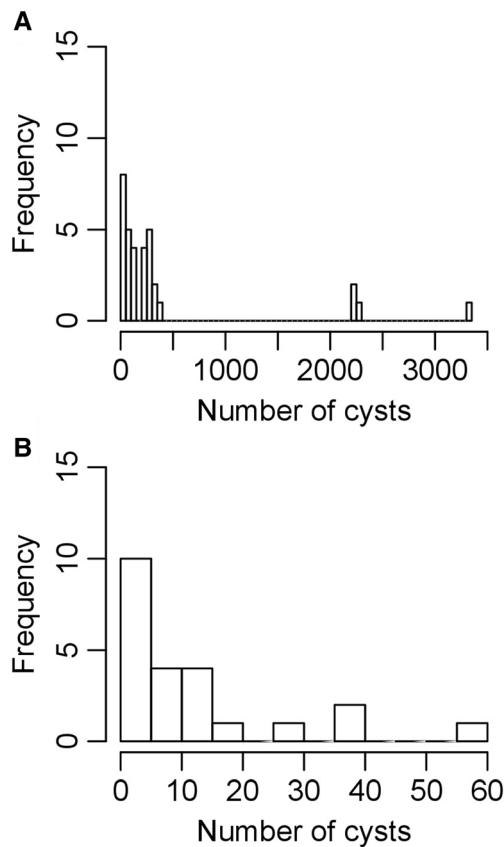


Fig. 1 Distribution of *M. oregonensis* infection intensity for all larval and adult **a** *Desmognathus quadramaculatus* ($N=69$) and **b** *Eurycea wilderae* ($N=160$). Note scale difference between species. Samples were pooled across all sampling sites

compared with *D. quadramaculatus* and *E. wilderae*. In part, the role of rare hosts in transmission dynamics will depend on whether the two more abundant species in the system are replaced by rare species when the rare species are present, or whether the overall number of salamanders is increased when the rare species are present (discussed in Wojdak et al. (2013)). In the first case, the overall number of hosts would not change, while in the second case, the rare species could actually increase the number of potential hosts in sites they inhabit.

Further evidence suggesting that life history differences are critical for infection is provided by the relationship between infection and body size. Body size as measured by snout-vent length (SVL) correlates strongly with age within a species of salamander (Bruce et al. 2002). When excluding the few individuals with over 2000 metacercariae, we found a positive relationship between body size and intensity of infection for *D. quadramaculatus*, which ranged in size from approximately 14 to 39 mm. Again, this may indicate that individuals are, in general, accumulating metacercariae over time, such that larger, older individuals, who have been exposed for longer periods, have higher numbers of metacercariae. Larger salamanders might also have more infection because there is more surface area available for cercariae to find and penetrate. For

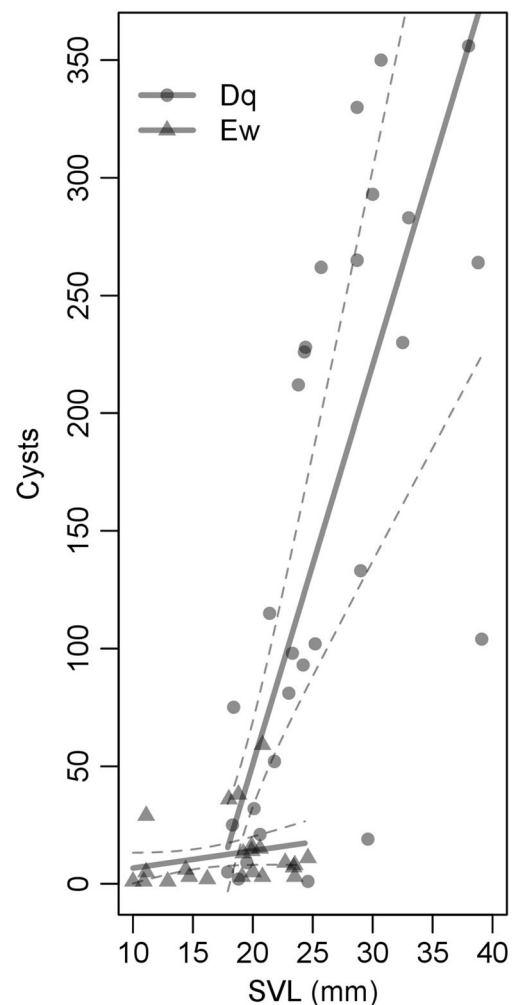


Fig. 2 The number of metacercariae found in *D. quadramaculatus* (circle) and *E. wilderae* (triangle). Points represent individual animals, thick lines are model predictions, and dashed lines are 95 % confidence intervals around the model predictions

instance, larval *E. wilderae* tend to be much smaller than *D. quadramaculatus* even when at similar ages, which make *E. wilderae* smaller overall targets for cercariae. Larger size classes are lacking for *E. wilderae* because they usually metamorphose into a more terrestrial adult after a single larval season (Bruce 1982). Since the exposure time of *E. wilderae* is limited by the short larval period, their infection level may also be more dependent on environmental factors, and even less on age or size. Life history traits, including larval duration, body size and habitat use, likely influence host exposure and ultimately infection intensity across host species.

Variability in infection level among potential hosts may have strong implications for transmission dynamics. Raccoons, the definitive host for this system, are nocturnal, generalist mesopredators that prefer to forage along forest edges and in wetlands, especially during the early spring and summer (Barding and Nelson 2008). They tend to forage using area-restricted searches and focus most of their efforts

in areas with shallow water and along linear stream and habitat boundaries (Byrne and Chamberlain 2012). Crayfish are a common and abundant prey item for raccoons in this habitat (Baker et al. 1945), but plethodontid salamanders are also abundant and are found in the same microhabitats in streams as crayfish. Because *D. quadramaculatus* is one of the most abundant salamander species and represents the largest portion of salamander biomass in the study streams (Milanovich 2010), they may also be relatively common prey for raccoons. If *D. quadramaculatus* is the primary second intermediate host in the transmission cycle, then variation in the abundance and infection intensity of *D. quadramaculatus* may be an important determinant of *M. oregonensis* distribution in the landscape.

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