



Larval wood frog (*Rana* [= *Lithobates*] *sylvatica*) development and physiology following infection with the trematode parasite, *Echinostoma trivolvis*

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ARTICLE INFO

Article history:

Received 15 September 2012

Received in revised form 11 December 2012

Accepted 13 December 2012

Available online 23 December 2012

Keywords:

Amphibian

Energetics

Metabolism

Oxygen consumption

Phenotypic plasticity

Respiration

Tadpole

ABSTRACT

Parasites can potentially affect host energetics through a variety of mechanisms including diverting energy from host functions or eliciting energetically costly responses. In many systems energetic costs of parasite infection remain poorly defined. The widespread trematode *Echinostoma trivolvis* can cause mortality of and pathology in larval amphibians. However, physiological impacts of *E. trivolvis* infection have received limited attention. To evaluate the effects of *E. trivolvis* on larval amphibian survival, growth and development, we studied a wide range of infection intensity in wood frog, *Rana* (= *Lithobates*) *sylvatica*, tadpoles in laboratory experiments and outdoor mesocosms. To assess potential underlying physiological costs of infection, we measured tadpole energetics and phenotypic plasticity of the intestines as a compensatory mechanism to offset increased energy costs. Survival was high in all tadpoles, but the highest infections decreased the growth and slowed the development of tadpoles raised in mesocosms and the laboratory. However, infections failed to elicit detectable energetic costs or phenotypic changes in intestinal size. The lack of energetic costs observed in our study emphasizes the complex and often context-dependent nature of energetic costs of parasitism and suggests that other mechanisms, such as changes in host behavior, may contribute to sub-lethal effects on growth and development.

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1. Introduction

An organism's energy resources can be allocated among the functions of maintenance, growth, storage, and reproduction (Sandland and Minchella, 2003). Parasite infection can divert energy resources from host functions through direct competition between parasite and host for energy or indirectly through energetically costly host responses to infection such as immune system stimulation (Kristan and Hammond, 2000; Sandland and Minchella, 2003). Physiological damage caused by parasite infection may also result in energetic consequences for the host (Khokhlova et al., 2002). However, the diversity of parasite–host interactions, including factors related to differences in host metabolism and parasite-induced pathology, prevents generalizations about energetic costs of parasitism (Robar et al., 2011). In some cases metabolic rate of hosts is significantly reduced (Vernberg and Vernberg, 1971; Kilgore et al., 1988; Connors and Nickol, 1991) while other cases show increases (Lester, 1971; Meakins and Walkey, 1975) or no response (Munger and Karasov, 1989). Identifying the conditions where energy costs are

significant is important because it provides a potential mechanism to translate physiological responses to changes in individual fitness, which may in turn affect populations (Lettini and Sukhdeo, 2010).

Understanding physiological costs of parasitism is relevant for amphibians, because their populations are declining worldwide due to numerous threats, including disease (Daszak et al., 2003). Trematode parasites have recently been investigated as sources of pathology and mortality, particularly for larval amphibians (Johnson and McKenzie, 2008). *Echinostoma trivolvis* is the most commonly reported echinostome trematode infecting amphibian intermediate hosts (Johnson and McKenzie, 2008). This parasite is known to cause mortality, edema, and decreased growth in laboratory-exposed amphibians (Beaver, 1937; Fried et al., 1997; Schotthoefer et al., 2003; Holland et al., 2007) and is suspected of reducing recruitment and influencing amphibian population dynamics (Beasley et al., 2005). Meanwhile, underlying physiological mechanisms of these effects have received only limited investigation (Martin and Conn, 1990; Holland et al., 2007; Orlofske et al., 2009).

The pathology induced by *E. trivolvis* suggests impairment of physiological processes and potential activation of compensatory mechanisms. *E. trivolvis* metacercariae, an encysted stage infecting the second intermediate host, are located in amphibian kidneys, organs that contribute significantly to amphibian standard metabolic rate (SMR; the basal daily metabolism of a post-absorptive, resting ectotherm, McNab, 2002; Steyermark et al., 2005). The occurrence of edema

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in echinostome-exposed amphibians implies kidney dysfunction caused by metacercariae-induced renal inflammation (McClure, 1919; Faeh et al., 1998). Kidney damage could provide a mechanism by which parasites affect host metabolic rates. It is also possible that *E. trivolvis* could directly contribute to elevated host metabolism through the additive effect of their own metabolism, but this may not require oxygen (Vernberg and Vernberg, 1971). Metacercariae have also frequently been considered a relatively metabolically inactive dormant stage, particularly those with rapid (<24 h) development inside the host (Vernberg and Vernberg, 1971; Fried, 1997). Parasite-infected hosts could compensate for increased metabolic demands by increased foraging or by phenotypic changes in intestinal morphology to increase the rate and efficiency of digestion (Kristan and Hammond, 2000; Schwanz, 2006). Tadpoles express significant intestinal size plasticity in response to predation and competition pressure (Relyea and Auld, 2004), but the plasticity of this organ in response to parasitism has only recently been considered (Orlofske et al., 2009).

We studied impacts of *E. trivolvis* infection in larval wood frogs, *Rana* (= *Lithobates*) *sylvatica*, using a combination of laboratory and mesocosm experiments. By examining a range of infection intensity, we expected to gain insight into the changes in physiology, growth, and development across a range of infections broadly applicable to natural infection levels. We predicted that our exposures would result in sublethal infections and that adverse effects on growth and development would be correlated with the number of encysted parasites. We also predicted that reduced growth and slowed development may result from increased metabolic demands caused by pathology. Thus, we quantified the metabolic costs of infection and phenotypic changes in intestinal size that might emerge as a compensatory means to increase digestive efficiency.

2. Materials and methods

2.1. Study system

Echinostome metacercariae have been documented in free-living *R. sylvatica* tadpoles (Najarian, 1955; McAllister et al., 1995; Woodhams et al., 2000) and have been used extensively to investigate various aspects of host–parasite interactions (Thiemann and Wassersug, 2000a,b; Belden, 2006; Koprivnikar et al., 2006; Toledo et al., 2007; Griggs and Belden, 2008; Johnson and McKenzie, 2008). The life cycle of *E. trivolvis* requires three hosts. The first intermediate host is the ubiquitous snail *Helisoma trivolvis* infected by free swimming miracidia that hatch from eggs deposited in definitive host feces (Schmidt and Fried, 1997). A broad diversity of second intermediate hosts can be infected by the second free-living stages (cercariae), including snails, and larvae and adults of several amphibian species (Huffman and Fried, 1990; Kanev et al., 1995). The definitive hosts of *E. trivolvis* are thought to include a variety of birds and mammals, which consume the infected second intermediate hosts (Johnson and McKenzie, 2008), although the current taxonomy of the North American echinostomes has recently been questioned based on molecular analyses (Detwiler et al., 2010). Across all species of larval amphibians, natural infection levels in larval amphibians range from 50 to 1600 metacercariae per larva (Fried and Bradford, 1997; Skelly et al., 2006). Specifically, infections of *R. sylvatica* with echinostome-like metacercariae were much lower, with the highest estimated average of only 90 metacercariae (Woodhams et al., 2000). *R. sylvatica* is the most widespread North American amphibian (Redmer and Trauth, 2005), ranging from boreal environments of Canada to the southern Appalachian Mountains in the United States, and are host to a wide array of parasite taxa (McAllister et al., 1995).

2.2. Parasite culture

In September 2007 and February 2008, we collected feces containing *E. trivolvis* eggs from laboratory-infected golden hamsters (*Mesocricetus*

auratus). Over the course of one evening each month, feces were collected in 120-mL of water using standard rodent metabolism cages. From this 120-mL collection, 10-mL aliquots were added directly to four plastic 4-L containers each containing 2-L of dechloraminated tap water (Aqua Safe®, Tetra, Blacksburg, VA, USA) and 10 uninfected, laboratory-raised *Helisoma trivolvis* snails (40 snails total). The number of eggs in the feces was not quantified for our snail exposures, although similar collections from these same hamsters during this time period yielded 666–1043 eggs/mL. The water containing hamster feces and snails was left undisturbed for three weeks to allow hatching of *E. trivolvis* eggs (Belden et al., 2009). Thereafter, 50-percent water changes were performed weekly. Snails were maintained at 22 °C and fed lettuce ad libitum supplemented with fish food (Tetra-Min, Tetra). Twelve weeks after the addition of *E. trivolvis* eggs, snails were screened for infection by individually warming snails under an incandescent light and microscopically examining the water for cercariae (Schmidt and Fried, 1996). A total of 27 infected snails were identified and subsequently maintained individually in 100 mL of water at 8–10 °C to prevent shedding and re-infection of the snails by metacercariae (Kuris and Warren, 1980).

2.3. Amphibian collection

Four *R. sylvatica* egg masses were collected on 22 February 2008 from an ephemeral pond in Montgomery County, Virginia and transported to the laboratory. Egg masses were transferred gradually from pond water to a 3:1 mix of dechloraminated (ChlorAm-X®, AquaScience Research Group, Inc., North Kansas City, MO, USA) tap water and well water (53.7 and 364 mg/L of CaCO₃ respectively). This mix of source waters was used to create an acceptable hardness level of 108 mg/L CaCO₃ and to match the water chemistry of the outdoor mesocosms described below.

2.4. Laboratory experiment

To investigate the physiological and developmental effects of *E. trivolvis* infection, tadpoles were exposed to a range of parasite intensities in the laboratory at 1, 2, or 3 time periods. In nature, second intermediate hosts accumulate infection during multiple transmission events (Ballabeni and Ward, 1993; Torchin et al., 2005), potentially producing continuous pathology as new parasites develop and encyst. Physiological responses of hosts to gradual infection can differ greatly from those experiencing a single inundation of parasites. Furthermore, it has been suggested that tadpoles can survive higher *E. trivolvis* infections through gradual accumulation than from single laboratory exposure (Griggs and Belden, 2008), which is a possible explanation for observations of high natural infections (Skelly et al., 2006). Therefore, we used a pulsed, gradual parasite exposure to closely mimic exposure in nature.

Sixty healthy *R. sylvatica* embryos with intact jelly coats were removed from each of the four clutches (240 embryos total) and maintained at 18 °C in a temperature-controlled environmental chamber (Adaptis, Conviron; Manitoba, Canada). On the day all eggs hatched, 2 March 2008, 64 hatchlings were chosen randomly from the mixed clutches and assigned to individual 4-L plastic containers containing 3 L of water. Prior to the experiment, tadpoles were provided with a 3:1 mixture of ground rabbit chow and fish food ad libitum. Throughout the experiment, tadpoles were weighed weekly by removing the tadpole from the container with a net, blotting excess moisture with tissue paper, and placing the tadpole in a tared cup of water. Rations equal to eight percent of each individual's body mass per day were provided three times a week. The rations were provided after 50-percent water changes.

We treated individual tadpoles as unique sampling units. Tadpoles were randomly assigned to three sampling periods corresponding to 1, 2, or 3 exposures to *E. trivolvis* cercariae occurring 18–19 d, 28–29 d, and 38–39 d post hatch. At the beginning of the experiment all tadpoles (18–19 d post hatch; n = 64) were exposed to one of eight treatments (0 control, 5, 9, 15, 36, 45, 60, or 75 cercariae) with eight tadpoles per

treatment. At 24–25 d post hatch, two tadpoles from each treatment were used for physiological measures and sacrificed to document encystment. The remaining tadpoles ($n = 6/\text{trt}$) were then exposed to the same number of cercariae a second time 28–29 d post hatch, attaining a cumulative exposure of 0, 10, 18, 30, 72, 90, 120, or 150 cercariae. Six days later, two tadpoles from each group were sampled again and the remaining tadpoles ($n = 4/\text{trt}$) were exposed to parasites for a third time 38–39 d post hatch, reaching cumulative exposure to 0 (control), 15, 27, 45, 108, 135, 180, or 225 cercariae. Two tadpoles from each treatment were sampled six days later. Remaining tadpoles were maintained until 50 d post-hatch when they were fasted for 72 h, euthanized and dissected to quantify encystment and for intestinal measurements. A mixture of cercariae from at least three snails was used for each exposure. Snails were induced to shed cercariae under a heat lamp. Cercariae were counted using a dissecting microscope and glass pipette, then dispensed into a 120-mL cup containing the tadpole in 40 mL of water and left undisturbed for 24 h. The container volume was chosen to discourage evasive tadpole movement and ensure successful encystment (Kiesecker, 2002; Taylor et al., 2004; Belden and Kiesecker, 2005). During each infection we exposed half of the tadpoles to cercariae on each of two consecutive days to maintain the same 6 day post-exposure timing for respirometry measurements (see below). Tadpoles had an average ± 1 SE mass of 266 ± 5 mg ($n = 64$), 415 ± 11.7 mg ($n = 46$), and 698 ± 26 mg ($n = 30$) at the first, second, and third exposures, respectively, and the range of developmental stages (Gosner, 1960) was: 26–27, 27–32, and 30–37, respectively. After each exposure, tadpoles were examined for mortality and edema, weighed, and returned to their individual containers. If edema was observed, tadpoles were examined every 12 h until recovery or death.

Respiration was quantified using closed-circuit respirometry (see methods below) 6 d after each cercariae exposure (i.e., 24–25 d, 34–35 d, 44–45 d post hatch). Two tadpoles from each treatment (16 total tadpoles) were measured over the course of each two-day sampling period corresponding to the two day infection periods. At the completion of each respirometry sampling period, individuals were euthanized with an overdose of MS-222 (tricaine methanesulfonate, ACROS Organics, Morris Plains, New Jersey) and immediately dissected. The wet and dry mass (nearest 0.1 mg) and length of intestines (nearest mm) were recorded. The entire kidney (pronephros, mesonephros and connecting Wolffian duct) was removed from each side of the tadpole and examined for encysted *E. trivolvis* metacercariae using a compound microscope.

2.5. Mesocosm experiment

To further evaluate how *E. trivolvis* cercariae would affect amphibians at a range of infection intensities, we used outdoor mesocosms with tadpoles exposed to cercariae shed from infected snails. Mesocosms consisted of eight 1500 L cattle watering tanks filled with 1000 L of 1:1 dechloraminated tap water:well water, 1 kg of dried leaf litter, and 17 g of ground rabbit chow (LM Animal Farms®, The Hartz Mountain Corporation, Secaucus, NJ, USA). Algal communities were initiated 11 d prior to tadpole introduction by adding 2 L of filtered (200- μm sieve) water from two local ponds. Each mesocosm was covered with nylon screening to prevent colonization by predators or competitors.

Thirty hatchling *R. sylvatica* were drawn from composites of four clutches of eggs and allocated to each of the eight mesocosms. The tadpoles were maintained for 40 days in the mesocosms until they reached size and stage suitable for exposure to cercariae (approximately 250 mg and Gosner stage 26–27). On 11 April, 2008, tadpoles in mesocosms were exposed to variable densities of cercariae in a regression-based experimental design by adding different numbers of laboratory-infected snails (1, 2, 3, 5, 6, or 7) directly to six of the mesocosms. The remaining two mesocosms served as controls; one contained no snails and the other contained 7 uninfected snails. Snails (mean mass ± 1 SE = $958 \pm$

66 mg) were randomly selected and acclimated to mesocosm conditions before they were released.

One randomly-selected tadpole was collected from each mesocosm at four time-periods (immediately prior to snail addition, and 8, 16, and 23 d after snail addition) to provide an approximation of the mass, developmental stage, infection status, and respiration rate of larvae in each treatment. In the final collection, four additional tadpoles were collected for a more robust comparison of final mass, developmental stage, and infection. Tadpoles remaining after these samples were collected at metamorphosis for other experiments (Orlofske et al. *unpubl. data*). The first collection provided pre-treatment respiration measurements and verified that tadpoles were not infected previously. Upon collection, tadpole wet mass was recorded to the nearest ± 0.1 mg after blotting with tissue paper to remove excess moisture. Tadpoles then were placed into individual containers, transported to the laboratory, and acclimated for 48 h through a series of 50-percent water changes in an environmental chamber maintained at 18 °C. After respiration rate measurements were completed, tadpoles were euthanized and dissected as described above. Tadpoles weighed an average ± 1 SE of 366 ± 25 mg ($n = 8$) and ranged from Gosner stage 26 to 27 immediately prior to snail addition. The masses and ranges of developmental stages at each collection were: at 8 d, 498 ± 18 mg ($n = 8$) and stages 28–30, at 16 d, 787 ± 24 mg ($n = 8$) and stages 31–34, and at 23 d, 928 ± 25 mg ($n = 40$) and stages 32–37.

2.6. Respirometry

Tadpole respiration rates (O_2 mL/h) were measured on a computer-controlled, indirect, closed-circuit respirometer (Micro-Oxymax, Columbus Instruments, Columbus, OH, USA) using techniques similar to those used for eastern mosquitofish (*Gambusia holbrooki*; Hopkins et al., 2003), southern toad tadpoles (*Bufo terrestris*; Beck and Congdon, 2003), bullfrog tadpoles (*Rana catesbeiana*; Rowe et al., 1998), and pickerel frog tadpoles (*Rana palustris*; Orlofske et al., 2009; Orlofske and Hopkins, 2009). The respirometer was calibrated prior to each trial using a certified gas mixture. Respirometry chambers consisted of 100-mL sealed glass culture bottles. Each chamber contained 80 mL of well oxygenated water and was maintained in an environmental cabinet at 18 °C. Oxygen consumption rates (mL/h) were monitored simultaneously in one control chamber containing a medical battery (Duracell Procell Zinc Air Medical DA 146, 8.4 V, Proctor & Gamble, Bethel, CT, USA) with a known rate of O_2 consumption, and one blank chamber filled with water. Each air sample was dried using a hygroscopic drier containing nafion tubing (Columbus Instruments) and adjusted for carbon dioxide (measured concurrently) prior to measuring tadpole respiration rates. Oxygen consumption was measured within the chambers every 66 minutes and was corrected for temperature and pressure. Normoxic conditions were maintained by completely refreshing the air within the headspace of chambers every 2.5 h. Each trial lasted 24 h and started at approximately the same start time (1100–1200 h) to control for the influence of natural circadian rhythms on tadpole respiration (Roe et al., 2004). Prior to each respirometry trial, tadpoles were fasted for 48 h to reduce metabolic contributions from digestion (Crowder et al., 1998).

From the respiration measurements collected, we estimated the standard metabolic rate (SMR) of each individual. Because spontaneous activity can bias estimates of SMR, O_2 consumption of each tadpole was plotted over time and examined for activity peaks prior to statistical analysis. After visually inspecting the plots, we discarded the first measurement of each sampling trial because some were inflated due to stress caused by handling before trials. Each remaining O_2 consumption measurement was ranked and we used the lowest quartile value as our estimate of SMR for each individual (Hopkins et al., 2004). Visual inspection of the plots revealed that this method effectively represented baseline oxygen consumption of each animal in our study.

2.7. Statistical analysis

Prior to analysis, data were tested to determine whether they met assumptions of parametric models. In some cases, \log_{10} -transformation was required to meet these assumptions. Values for SMR and mass were \log_{10} -transformed because metabolism is a power function of mass (Chappell et al., 1996). The masses of fasted tadpoles were used in all analyses including mass. All statistical tests were conducted using JMP 7.0 (SAS Institute, Cary, NC, USA), and statistical significance was assessed at $\alpha = 0.05$.

In the laboratory experiment, we examined the relationship between cercariae exposure and encystment by regressing the number (\log_{10} transformed) and proportion of metacercariae recovered after each exposure against the total number of cercariae to which tadpoles were exposed. In addition, we tested whether the relationship was non-linear by fitting a non-linear regression and comparing the two models qualitatively using R^2 . To determine the relationship between the average percentage of metacercariae recovered from each treatment level (excluding controls) at each exposure, we used a one-way ANOVA. A second one-way ANOVA was performed to compare the average percent metacercariae recovered from tadpoles surviving to the end of the experiment among all treatment levels. To assess whether infection influenced growth, growth rate (mg/day) at each sampling period was regressed against the number of metacercariae recovered from each tadpole. To examine the effect of infection on development, the developmental stage at each sampling period was regressed against the number of metacercariae. The influence of infection on SMR ($\text{mL O}_2/\text{h}$) during each separate respirometry-sampling period was determined using multiple linear regression with the number of metacercariae and tadpole mass as independent variables. Tadpole intestinal wet mass (mg), dry mass (mg), and length (mm) were all highly correlated ($P < 0.0001$) so we only examined the relationship between tadpole intestinal wet mass through multiple linear regression with number of metacercariae and tadpole mass as independent variables in the model.

The mesocosm experiment was a regression design, intended to examine the effects of a broader range of metacercariae exposures than in our lab study. We used a regression design because they are a more powerful approach than ANOVA for a given number of experimental units, even without replication at each level of the regression (Cottingham et al. 2005). In all statistical comparisons, the mesocosm was treated as the experimental unit. Thus, when more than one tadpole was sampled from a mesocosm the mean response of tadpoles removed from each mesocosm was used in the analysis. Tadpole mass was regressed against the number of metacercariae recovered. The relationship of infection and development was assessed by regressing developmental stage against the number of metacercariae recovered. The influence of metacercarial infection on SMR ($\text{mL O}_2/\text{h}$) was determined using multiple linear regression with number of metacercariae and tadpole mass as independent variables. As in the laboratory experiment, we analyzed tadpole intestinal wet mass (mg) by multiple linear regression with number of metacercariae and tadpole mass as independent variables in the model because wet mass (mg), dry mass (mg), and length (mm) were all highly correlated ($P < 0.0001$). For the final collection, mean encystment number was regressed against the number of snails initially added to each mesocosm.

3. Results

3.1. Laboratory experiment

After the first exposure to cercariae, 17 (27%) tadpoles developed generalized edema in the treatments receiving 15–75 cercariae. The duration of edema ranged from 48 to 180 h, with an average of 71 ± 9 h until recovery. Edema was not observed after the second and third exposures. Four tadpoles receiving 5–60 cercariae died after the first exposure to cercariae, with a range of 3–15 metacercariae recovered

post-mortem. Two of these four tadpoles died during the 48-h fast prior to the first respirometry measurements. One control tadpole was excluded from all analyses because of abnormal oral disk morphology that may have interfered with feeding and growth. No mortality occurred after the second or third exposures to cercariae.

The number of metacercariae recovered was positively correlated with the number of cercariae to which tadpoles were exposed during all three exposure periods: first ($F_{1,13} = 18.86$, $R^2 = 0.61$, $P = 0.001$), second ($F_{1,14} = 14.58$, $R^2 = 0.53$, $P = 0.002$), and third ($F_{1,15} = 52.69$, $R^2 = 0.79$, $P < 0.0001$). The combination of data from all three time-periods showed a positive, non-linear relationship between the number of cercariae to which tadpoles were exposed and the number of metacercariae recovered ($F_{1,54} = 60.27$, $R^2 = 0.69$, $P < 0.0001$, Fig. 1). The proportion of cercariae recovered as metacercariae was not significantly related to the number of cercariae to which tadpoles were exposed after the first ($F_{1,11} = 0.35$, $R^2 = 0.03$, $P = 0.570$) or second ($F_{1,13} = 1.79$, $R^2 = 0.13$, $P = 0.206$) exposures. Interestingly, following the third exposure, there was a marginally significant, positive relationship between proportion encystment and the total cumulative number of cercariae exposed ($F_{1,12} = 4.38$, $R^2 = 0.26$, $P = 0.064$). However, across all exposure levels, the average percent encystment did not differ significantly among exposure periods (Table 1; $F_{2,39} = 0.299$, $P = 0.744$).

For the final surviving tadpoles ($n = 14$), a positive relationship was found between the number of cercariae exposed and the number of recovered metacercariae ($F_{1,13} = 14.82$, $R^2 = 0.55$, $P = 0.002$). Proportion encystment for these animals was not related to the total number of cercariae exposed ($F_{1,11} = 1.30$, $R^2 = 0.11$, $P = 0.282$). The average percentage of metacercariae recovered from these tadpoles was the lowest of all the time periods measured but did not differ significantly from those of any other infection period (Table 1; $F_{3,51} = 0.430$, $P = 0.732$). The percentage of melanized cysts was 42% in the final surviving tadpoles compared to 65% for tadpoles sampled closer to the third infection, suggesting that melanized cysts, dark brown cysts potentially containing dead metacercariae (Martin and Conn, 1990), were being gradually cleared by the host immune system.

Growth rate was not significantly affected by the number of metacercariae encysted following the first ($F_{1,13} = 1.81$, $R^2 = 0.13$, $P = 0.204$, Fig. 2a) or second exposure ($F_{1,14} = 3.12$, $R^2 = 0.19$, $P = 0.101$, Fig. 2b); however, growth rate of the tadpoles from the third exposure was negatively correlated with the number of metacercariae ($F_{1,15} = 6.61$, $R^2 = 0.32$, $P = 0.022$, Fig. 2c). In contrast, the growth rate for the final surviving tadpoles 12–13 days after the third exposure was not significantly related to the number of metacercariae encysted ($F_{1,13} =$

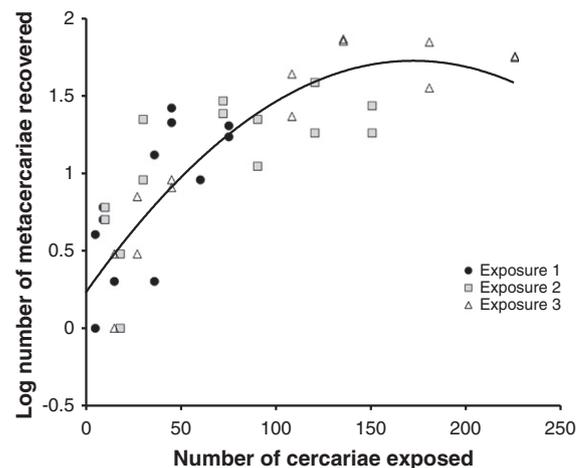


Fig. 1. Combined regression plot of \log_{10} -transformed number of *Echinostoma trivolvis* metacercariae recovered from laboratory raised *Rana* (= *Lithobates*) *sylvatica* tadpoles by total number of cercariae to which tadpoles were exposed at 18–19 (exposure 1), 28–29 (exposure 2), or 38–39 (exposure 3) d post hatch ($R^2 = 0.69$, $P < 0.0001$, for all three groups combined).

Table 1

Mean (\pm standard error) and range of metacercariae percent encystment, mass, and standard metabolic rate (SMR) of *Rana* (= *Lithobates*) sylvatica tadpoles after laboratory exposure to *Echinostoma trivolvis* cercariae at 18–19 d, 28–29 d, and 38–39 d post-hatch and at the end of the study (50 d).

Exposure	N	Percent encystment (\pm SE) ^a	Range of percent encystment ^a	Mass (mg) \pm SE	SMR (mL/h) \pm SE
First (18–19 d)	14	30.2 \pm 6.3	0.0–60.0	333 \pm 21	0.037 \pm 0.003
Second (28–29 d)	15	26.9 \pm 5.0	0.0–70.0	506 \pm 26	0.052 \pm 0.004
Third (38–39 d)	16	24.6 \pm 4.1	0.0–52.6	771 \pm 47	0.078 \pm 0.006
Final survivors (50 d)	14	22.3 \pm 4.7	1.9–51.7	900 \pm 43	N/A

^a Excluding controls.

0.01, $R^2 < 0.01$, $P = 0.909$). Developmental stage was not affected by the number of metacercariae recovered following the first ($F_{1,13} = 0.83$, $R^2 = 0.07$, $P = 0.378$), or third ($F_{1,15} = 3.26$, $R^2 = 0.19$, $P = 0.093$) exposures, or the final surviving tadpoles ($F_{1,13} = 0.12$, $R^2 = 0.01$, $P = 0.728$). Following the second exposure, however, there was a marginally significant negative relationship between the number of metacercariae recovered and developmental stage ($F_{1,14} = 4.69$, $R^2 = 0.26$, $P = 0.050$).

Both the mean mass and SMR of tadpoles increased during the experiment (Table 1). The number of metacercariae encysted was not significantly related to tadpole SMR at any of the sampling times ($P \geq 0.526$). As expected, there was a strong positive relationship between tadpole mass and SMR at each of the sampling times (in all cases $P \leq 0.008$).

Tadpole intestinal length increased during the course of the experiment ranging from 95 ± 5 mm at 18–19 days post-hatch to 123 ± 5 mm at 50 days post hatch. Likewise wet intestinal mass increased from 37 ± 4 mg to 78 ± 5 mg, and dry mass increased from 3.7 ± 0.4 mg to 8.3 ± 0.5 mg. Tadpole body mass was positively correlated with wet mass ($P \leq 0.05$), but the number of metacercariae recovered did not influence tadpole intestinal wet mass at any of the sampling times ($P \geq 0.208$).

3.2. Mesocosm experiment

Our regression-based experimental design was effective at generating a large range of metacercarial encystment in tadpoles. The mean encystment varied from a low 0.6 metacercariae per tadpole in the mesocosm with one infected snail to 285.8 metacercariae per tadpole in the mesocosm with seven infected snails. The average number of encysted metacercariae among mesocosms was positively correlated to the number of infected snails initially added to each mesocosm ($F_{1,7} = 7.55$, $R^2 = 0.56$, $P = 0.033$). The first collection of tadpoles from the mesocosms verified that tadpoles were not previously infected. No metacercariae were recovered from tadpoles in control mesocosms without snails or from mesocosms containing seven uninfected snails.

The cumulative effect of exposure to parasites in the mesocosms was similar to what we observed in the laboratory. Tadpole mass was not related to the number of metacercariae recovered from tadpoles in the first ($F_{1,7} = 0.00$, $R^2 = 0.00$, $P = 0.990$) or second ($F_{1,7} = 1.93$, $R^2 = 0.24$, $P = 0.214$) collections following addition of infected snails. However, there was evidence of a negative relationship between the number of metacercariae recovered and the mass of tadpoles selected for respirometry measurements following the third collection ($F_{1,7} = 5.66$, $R^2 = 0.49$, $P = 0.055$). This negative effect of metacercariae on tadpole mass was clearly evident when the mean of five individuals was used for each mesocosm ($F_{1,7} = 12.17$, $R^2 = 0.67$, $P = 0.013$, Fig. 3). Tadpole mass was similar between the control mesocosm without snails (915 ± 42 mg SE) and the control mesocosm with seven uninfected snails (864 ± 43 mg SE).

Tadpole developmental stage was not related to the number of metacercariae encysted after the first collection ($F_{1,7} = 0.19$, $R^2 = 0.03$, $P = 0.681$) or final collections ($F_{1,7} = 0.13$, $R^2 = 0.02$, $P = 0.733$), even considering the mean of five individuals from each mesocosm ($F_{1,7} =$

3.59, $R^2 = 0.37$, $P = 0.107$). However, after the second collection developmental stage was negatively related to the number of metacercariae encysted ($F_{1,7} = 29.47$, $R^2 = 0.83$, $P = 0.002$).

Tadpole SMR ranged from 0.027 ± 0.001 mL/h SE at the first collection to 0.054 ± 0.003 mL/h SE at the final collection and was not significantly related to the number of metacercariae recovered at any collection ($P \geq 0.430$). The SMR of tadpoles was not significantly related to tadpole mass in most cases ($P \geq 0.144$), with the exception of the third collection ($P = 0.023$). This was likely attributable to the narrow mass ranges within each sampling period (first (254–404 mg), second (360–565 mg), third (575–702 mg), final (624–992 mg)) and small sample sizes ($n = 8$). Indeed, a regression analysis of all tadpoles ($n = 32$) sampled during the study (254–992 mg) revealed a strong effect of mass on SMR ($R^2 = 0.77$, $P < 0.0001$).

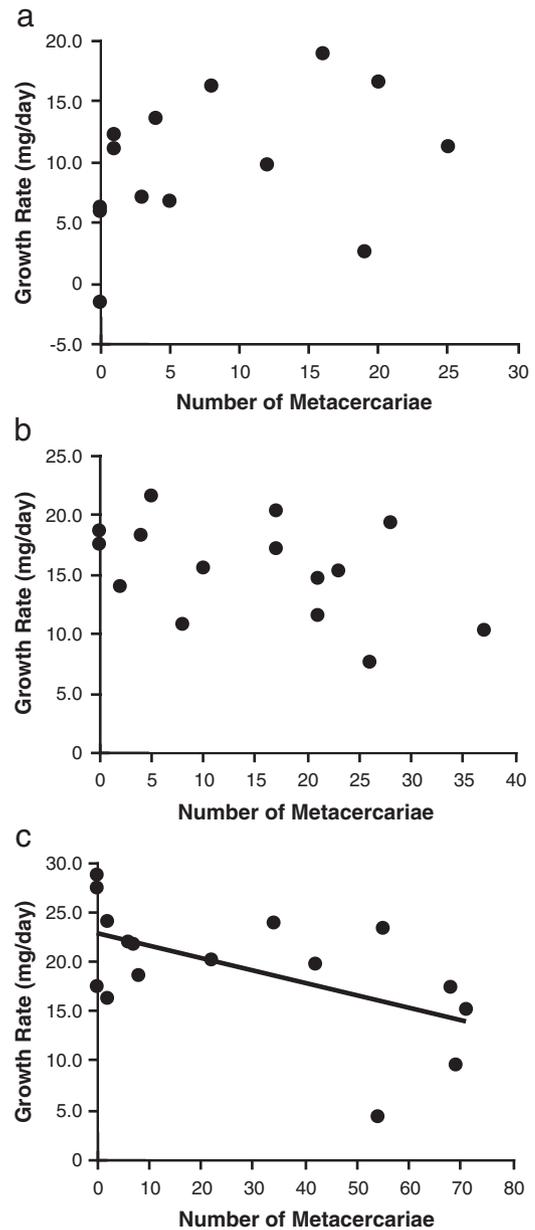


Fig. 2. Growth rate (mg/day) of individual laboratory-raised *Rana* (= *Lithobates*) sylvatica tadpoles as a function of number of *Echinostoma trivolvis* metacercariae recovered after: a. first exposure (18–19 d post hatch), b. second exposure (28–29 d post hatch), and c. third exposure ($R^2 = 0.32$, $P = 0.022$, 38–39 d post hatch). Note different scales of x and y-axes.

All variables associated with tadpole intestinal morphology increased over the experiment. Intestinal length increased from 93 ± 5 mm to 145 ± 3 mm at the end of the experiment. Likewise wet intestinal mass increased from 39 ± 2 mg to 89 ± 3 mg, and dry mass increased from 4.0 ± 0.2 mg to 9.0 ± 0.4 mg. Intestinal wet mass was not significantly related to the number of metacercariae recovered at any sampling period ($P \geq 0.554$, $n = 8$). However, tadpole mass was significantly related to intestinal wet mass at the first two sampling periods ($P \leq 0.017$), but not the second two ($P \geq 0.365$).

4. Discussion

Our study assessed the physiological effects of a range of parasite infection as a possible mechanism for reduced growth and slower development. We used a multiple infection procedure in our laboratory study and fostered a gradual accumulation of infection in the mesocosm experiment to create a wide range of ecologically representative infection intensities. Using both approaches, we were able to non-lethally infect tadpoles with parasite burdens comparable to those reported in nature (Fried and Bradford, 1997; Woodhams et al., 2000). We observed cumulative negative effects of parasites on growth and development, but we found no evidence of altered metabolic rates as an underlying physiological mechanism or of compensatory phenotypic changes in intestinal size.

Our results support previous studies that demonstrated that the consequences of infection are dependent on the developmental stage and age of larval amphibians. In general, young tadpoles at early developmental stages experience the highest mortality and greatest growth reduction (Fried et al., 1997; Schotthoefer et al., 2003; Holland et al., 2007) but tadpoles at later developmental stages (e.g., Gosner stage > 25) appear more resistant to the effects of infection (Schotthoefer et al., 2003; Holland et al., 2007). Our initial laboratory infection resulted in edema accompanied by limited mortality when tadpoles were still within the range of developmental stages and ages most vulnerable to infection (Fried et al., 1997; Schotthoefer et al., 2003; Holland et al., 2007). However, no edema or mortality was observed as the tadpoles grew and surpassed Gosner stage 27.

In contrast to earlier studies (Schotthoefer et al., 2003; Holland et al., 2007), there was no significant influence of the sampling time on average percent of metacercariae recovered across all exposure levels, suggesting that neither developmental stage nor larval body size at

time of exposure strongly influenced infection success. This could be explained by species and body size differences among the studies or by our infection procedure where we exposed tadpoles to parasites at repeated intervals, so that at each infection interval tadpoles gradually progressed in developmental stages and infection burdens as they would in nature. Recent evidence suggests that encystment rates may be changing over the course of larval development as a result of cyst degradation by the host immune response (Martin and Conn, 1990; Belden, 2006; Holland, 2009); however we cannot directly compare our study with the literature because we repeatedly exposed the same individuals whereas other studies used single infections. We frequently observed melanized cysts, dark brown cysts potentially containing dead metacercariae (Martin and Conn, 1990), suggesting that they persist for long time periods, up to several weeks, even in tadpoles infected at later stages and ages. However, the decline in the percentage of melanized cysts from the third laboratory exposure until the end of the experiment could be a result of melanized cysts being gradually degraded by the host immune system.

We observed reduced growth and slower development in response to *E. trivolvis* infection in the laboratory experiment; however, these responses emerged gradually. Effects on development were evident at the second exposure when stages ranged from 27 to 32 (Gosner, 1960). The consequences of cumulative infection could correspond to interference of encysted metacercariae with kidney development (Fox, 1961; Viertel and Richter, 1999; Schotthoefer et al., 2003). Differences in growth were detectable only after the third exposure to cercariae. This observation suggests that high parasite burdens were necessary to affect growth. In addition, we also found evidence of potential recovery when further infection ceased. For the final group of surviving tadpoles (12 days after their last infection) no effect of metacercariae on growth was detected, perhaps as cysts were eliminated by the immune system (Holland, 2009). Although the effects on growth and development we observed were short lived due to recovery, in a natural situation decreased growth rates could translate to a longer developmental period and hence exposure to more parasites or other threats (Raffel et al., 2010).

Tadpoles collected from the mesocosms exhibited high parasite burdens after more than three weeks of exposure. Infections were strongly associated with the number of cohabitating-infected snails, supporting the inference that higher infected snail density can increase infection levels (Johnson et al., 2007). Given that we only subsampled a fraction of individuals with each mesocosm, caution should be used in interpreting our results. However, we assume that our subsampling was representative of all individuals in each mesocosm. In spite of our sampling constraints, the number of metacercariae recovered from tadpoles in the mesocosm with 7 infected snails was more than 3 times the number recovered from any of our laboratory exposures, demonstrating the effectiveness of this approach to produce high infection levels. This observation provides further evidence that gradual accumulation allows tadpoles to accommodate higher infections, which may explain high infection levels observed in nature (Thiemann and Wassersug, 2000a; Griggs and Belden, 2008).

In the mesocosm experiment, the effects on growth and development were also only apparent after cumulative infection, but again, we did not extensively sample within tank variation, so some caution is warranted. We did see that the mass of tadpoles after three weeks of parasite accumulation was negatively correlated with the number of encysted metacercariae. Lower growth rates could be attributed to reaching a threshold infection level after gradual exposure (Goater et al., 1993, 2005; Franz and Kurtz, 2002; Collyer and Stockwell, 2004), or to sufficient time from the initial infection during the earliest developmental stages for growth rates among infected individuals to diverge significantly (Goater et al., 1993; Fried et al., 1997). Similar to the laboratory study, effects on development corresponded to stages 31–34 and were only apparent at a single sampling point that may correspond with particular development changes in the tadpole kidneys. Our results

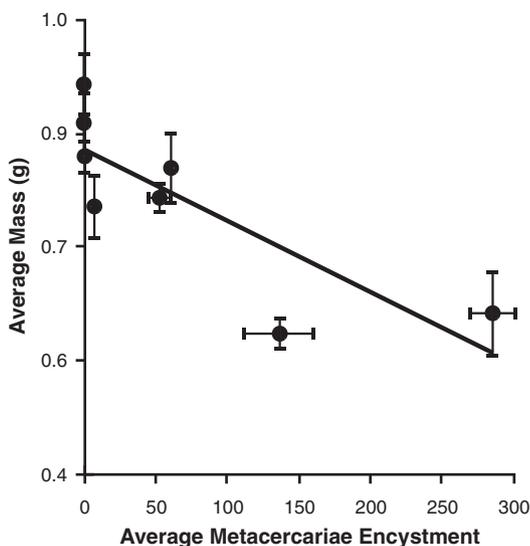


Fig. 3. The average mass \pm SE (g) ($n = 5$) of *Rana* (= *Lithobates*) *sylvatica* tadpoles collected from individual mesocosms 23 days after addition of 1–7 of *Echinostoma trivolvis*-infected snails as a function of mean number of metacercariae \pm SE encysted in tadpoles in each mesocosm ($R^2 = 0.67$, $P = 0.013$).

suggest that growth and development may be impacted in natural populations where high infections, similar to those achieved in our mesocosm experiment, can ultimately occur. However, other mesocosm studies have shown no effects of *E. trivolvis* on growth and development (Belden, 2006; Koprivnikar et al., 2008) or only effects on development (Raffel et al., 2010).

Despite the initial appearance of edema and subsequent effects on growth and development, there was no effect of metacercariae infection on tadpole metabolic rate in either the lab or mesocosm experiment, even at the highest levels of infection observed in the mesocosms. These results are consistent with recent work that found no energetic costs during trematode encystment in *Rana* (= *Lithobates*) *palustris* tadpoles (Orlofske et al., 2009). Because ample resources were available to tadpoles in both studies, the lack of a detectable effect on metabolism suggests that other disruptions must lead to reductions in growth and developmental rate. For example, changes in feeding behavior or activity are well known responses in parasitized animals (Cunningham et al., 1994) and could compromise energy acquisition and utilization. Although baseline metabolism (e.g., SMR) is not influenced by these parasites, it is possible that energetic consequences of parasite infection may be evident only during activity (Lester, 1971; Meakins and Walkey, 1975; Chappell et al., 1996) or in conjunction with temperature stress (Hayworth et al., 1987; Connors and Nickol, 1991; Booth et al., 1993; Kristan and Hammond, 2000; Meagher and O'Connor, 2001), or other energetically demanding conditions.

We also hypothesized that if tadpoles were energetically challenged by parasite exposure they would respond by modifying their gut morphology to increase digestive efficiency. However, tadpole intestinal morphology did not consistently respond to the presence of metacercariae. Similarly, a recent study found no changes in intestinal size in *R. palustris* tadpoles exposed to *E. trivolvis* (Orlofske et al., 2009). Although both of these studies suggested that tadpoles under different conditions do not modify their gut morphology in response to this parasite, we offer an important consideration for future work. Abundant food resources provided to tadpoles in the laboratory and low densities of tadpoles in mesocosms may have eliminated the need to develop a larger, more efficient digestive system (Franz and Kurtz, 2002).

In conclusion, our study shows that realistic levels of *E. trivolvis* infection can reduce growth and development in larval amphibians without subsequent physiological costs. Understanding energetic costs of parasitism is particularly important for understanding interactions between *E. trivolvis* metacercariae and larval amphibians, which have become an important model for studying how anthropogenic change (Belden, 2006), environmental contamination (Thiemann and Wassersug, 2000a; Koprivnikar et al., 2007; Griggs and Belden, 2008; Rohr et al., 2008), ecological interactions (Rohr et al., 2009; Belden and Wojdak, 2011) and disease emergence (Skelly et al., 2006; Rohr et al., 2008) influence parasite-host systems. More closely examining the physiological effects of *E. trivolvis* infections to accurately determine mechanisms underlying pathology is critical for evaluating the impact of this widespread parasite on free-living amphibian populations.

Acknowledgments

The help of R.C. Jadin in setting up mesocosms and collecting amphibian eggs is greatly appreciated. The authors also thank P.D. Widder, J.M. Orlofske, C. Orlofske, and R.C. Jadin for comments on the manuscript. This research was approved by the Virginia Tech Institutional Animal Care and Use Committee (#07-021-FIW). This study was supported by the Department of Fish and Wildlife Conservation at Virginia Tech, a National Science Foundation grant (W.A.H., IOB-0615361), a Deans Diversity Fellowship through the Multicultural Academic Opportunities Program at Virginia Tech, and a Grant-In-Aid of Research from Sigma Xi to S.A.O. This material is based upon work supported by a National Science Foundation Graduate Research Fellowship (S.A.O., DGE 0707432).

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