Effects of *Echinostoma trivolvis* metacercariae infection during development and metamorphosis of the wood frog (*Lithobates sylvaticus*)

Sarah A. Orlofske \(^a\), Lisa K. Belden \(^b\), William A. Hopkins \(^a\)

---

A R T I C L E   I N F O

Article history:
Received 29 April 2016
Received in revised form 28 July 2016
Accepted 5 August 2016
Available online 17 August 2016

Keywords:
Energy
Parasite
Tadpole
Trematode
Oxygen consumption
Respiration
Metamorphic climax
Metabolism

A B S T R A C T

Many organisms face energetic trade-offs between defense against parasites and other host processes that may determine overall consequences of infection. These trade-offs may be particularly evident during unfavorable environmental conditions or energetically demanding life history stages. Amphibian metamorphosis, an ecologically important developmental period, is associated with drastic morphological and physiological changes and substantial energetic costs. Effects of the trematode parasite *Echinostoma trivolvis* have been documented during early amphibian development, but effects during later development and metamorphosis are largely unknown. Using a laboratory experiment, we examined the energetic costs of late development and metamorphosis coupled with *E. trivolvis* infection in wood frogs, *Lithobates [=Rana] sylvaticus*. *Echinostoma* infection intensity did not differ between tadpoles examined prior to and after completing metamorphosis, suggesting that metacercariae were retained through metamorphosis. Infection with *E. trivolvis* contributed to a slower growth rate and longer development period prior to the initiation of metamorphosis. In contrast, *E. trivolvis* infection did not affect energy expenditure during late development or metamorphosis. Possible explanations for these results include the presence of parasites not interfering with pronephros degradation during metamorphosis or the mesonephros compensating for any parasite damage. Overall, the energetic costs of metamorphosis for wood frogs were comparable to other species with similar life history traits, but differed from a species with a much shorter duration of metamorphic climax. Our findings contribute to understanding the possible role of energetic trade-offs between parasite defense and host processes by considering parasite infection with simultaneous energetic demands during a sensitive period of development.

© 2016 Elsevier Inc. All rights reserved.

---

1. Introduction

Fundamental to understanding animal physiology is the concept of energetic trade-offs among the competing processes of growth, development, maintenance and reproduction (Sterns, 1989; Roff, 2001; Zera and Harshman, 2001; Lee, 2006). Among the multitude of physiological costs inherent to self-maintenance, immune defense against parasite infection is thought to be particularly costly (Martin et al., 2003; Lee, 2006; Hawley and Altizer, 2010). For example, basal metabolic rate of Collared Doves (*Streptopelia decaocto*) increased by a maximum of 8.5% in response to challenge by a novel antigen, corresponding with antibody production (Eraud et al., 2005). However, there are relatively few studies quantifying the metabolic costs of immune challenge in wild vertebrate species, especially in response to parasites (Lochmiller and Deerenberg, 2000; Hawley et al., 2012). Costs of parasite defense are not limited solely to support of the immune system. They can also consist of repairing tissue damage, and can result from competition between the parasite and host for energy resources (Kristan and Hammond, 2000; Khokhlova et al., 2002; Sandland and Minchella, 2003).

Defense against parasites may elicit trade-offs with other functions or activities that require common resources, thereby influencing an animal’s fitness (Lee, 2006; Hawley and Altizer, 2010). For example, wood frog tadpoles exposed to ranavirus showed elevated corticosterone, which was associated with a more rapid progression through metamorphosis at the expense of body weight and immune responses (Warne et al., 2011). This illustrates how intrinsic factors, such as particular developmental periods, require increased energy allocation. This can potentially limit the investment in other processes, such as immunity, and lead to increased fitness-related consequences of infection.
Using this resource allocation framework helps explain why there may not be trade-offs between parasite defense and other demands unless they share required resources, occur simultaneously, or if available resources are insufficient to fuel competing demands (Lee, 2006; Hawley et al., 2012). Across a variety of host-parasite systems energetic costs of parasite infection were only evident or additive when there were competing energetic demands, such as during maximum activity, stress, or during mammalian pregnancy or lactation (Lester, 1971; Meakins and Walkey, 1975; Hayworth et al., 1987; Munger and Karasov, 1989; Connors and Nickol, 1991; Booth et al., 1993; Chappell et al., 1996; Meagher and O’Connor, 2001; Kristan and Hammond, 2000, 2003; Hawley et al., 2012; Novikov et al., 2015). Therefore, it is important to investigate energetic costs of parasitism during periods of elevated energy demand to determine the overall impact of parasites on hosts (Robar et al., 2011; Warne et al., 2011).

Larval amphibians and trematode parasites have become a model system for investigating many aspects of host-parasite interactions and could be used specifically to test physiological trade-offs of parasite defense and development (Warne et al., 2011; Blaustein et al., 2012; Koprivnikar et al., 2012). Echinostoma trivolvis is a widespread digenetic trematode infecting larvae of several amphibian species as intermediate hosts and occasionally causing mortality and reduced growth, especially in very small larvae (Beaver, 1937; Fried et al., 1997; Schottofer et al., 2003; Belden, 2006; Holland et al., 2007; Belden and Wojdak, 2011). Specifically, *E. trivolvis* metacercariae infect amphibian kidneys, causing renal inflammation, which can result in physiological dysfunction and edema (McClure, 1919; Faeh et al., 1998). Little is known about the immune response of larval amphibians to helminths, such as trematodes (Holland, 2009; Koprivnikar et al., 2012). However, *E. trivolvis* infection in amphibians is associated with granuloma formation, granulocyte infiltration, and a shift in the abundance and types of circulating leukocytes (Martin and Conn, 1990; Holland et al., 2007). Although some previous investigations of *E. trivolvis* infection in larval amphibians revealed reductions in growth with likely energetic underpinnings, no significant effects on host metabolic rate have been detected (Fried et al., 1997; Schottofer et al., 2003; Orlofske et al., 2009, 2013). However, it is possible that effects to host metabolism may become evident during developmental periods that are more demanding, such as amphibian metamorphosis (Warne et al., 2011; Blaustein et al., 2012).

Studies of amphibian metamorphosis indicate that this is an energetically demanding period where total energetic costs and developmental costs are significant (*Hoplobatrachus (= Rana) tigrinus*, Pandian and Marian, 1985; *Anaxyrus (= Bufo) terrestris*, Beck and Congdon, 2003; and *Lithobates palustris*, Orlofske and Hopkins, 2009). Compensatory responses of hosts to parasites could be limited during metamorphosis because of reliance upon stored energy resources (Duellman and Trueb, 1986; Beck and Congdon, 2003) and the potential ecological vulnerabilities imposed by delayed metamorphosis (Wassersug and Sperry, 1977; Arnold and Wassersug, 1978; Downie et al., 2004).

Here, we examine the energetic costs of parasite infection concurrent with amphibian metamorphosis, as well as characterize the energetic costs of metamorphosis in wood frogs (*Lithobates sylvaticus*). We used a laboratory experiment to create a range of *E. trivolvis* metacercariae infection in amphibian hosts. We assessed the fate of metacercariae encysted within the pronephros or larval kidneys after completion of metamorphosis. While *Lithobates clamitans* tadpoles can eliminate echinostome metacercariae according to age-dependent process (Holland, 2009), it is unknown whether metacercariae are shed during, or interfere with, the restructuring of the amphibian kidneys during metamorphosis. We predicted high survival given our realistic, gradual exposure procedure (as in Orlofske et al., 2013), but reduced growth and longer development time associated with infection intensity due to increased metabolic costs of infection. We predicted elevated total and developmental energy costs, longer period of metamorphic climax, and smaller size after completing metamorphosis accompanying *E. trivolvis* infection. Finally, we investigated the role of duration of metamorphic climax and body size on the developmental costs and total costs of amphibian metamorphosis.

### 2. Materials and methods

#### 2.1. Study system

*Echinostoma trivolvis* is a model parasite used frequently to investigate 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). *Echinostoma trivolvis* requires three hosts to complete its life cycle. The first intermediate host is the ubiquitous snail *Planorbella trivolvis* which is infected by free swimming miracidia that hatch from eggs deposited in definitive host feces (Schmidt and Fried, 1997). A wide array of second intermediate hosts can be infected by the second free-living stage (cercariae), including snails, and larvae and adults of several amphibian species (Huffman and Fried, 1990; Kaney et al., 1995). The definitive hosts include a variety of birds and mammals, particularly muskrats, which consume the infected second intermediate hosts (Johnson and McKenzie, 2008; Detwiler et al., 2012).

Wood frogs (*Lithobates [= Rana] sylvaticus*) are the most broadly distributed amphibian in North America (Redmer and Trauth, 2005) and are host to a diversity of adult and larval parasites (McAllister et al., 1995). One of the most commonly documented trematodes of wild *L. sylvaticus* tadpoles is *Echinostoma trivolvis* (Najarian, 1955; McAllister et al., 1995; Woodhams et al., 2000). In *L. sylvaticus*, natural infections with echinostomes averaged 90 metacercariae per host (Woodhams et al., 2000).

#### 2.2. Parasite culture

Methods for obtaining infected snails follow Orlofske et al. (2013). Briefly, *Echinostoma trivolvis* eggs were collected by mixing feces from laboratory-infected golden hamsters (*Mesocricetus auratus*) with a small amount of water, and adding it to containers with laboratory-raised *Planorbella trivolvis* snails. We did not quantify the number of eggs in the feces dilution, but similar collections from the same hamsters yielded 666–1043 eggs/mL. Water in the snail containers was left undisturbed for 3 weeks to allow for hatching of *E. trivolvis* eggs (Belden et al., 2009). We maintained snails for 3 weeks at room temperature with lettuce and flake fish food provided ad lib and 50% water changes performed weekly. We screened snails for infection by placing them in individual containers warmed with an incandescent bulb and microscopically examined the water for cercariae (Schmidt and Fried, 1996). After we confirmed parasite infection, we maintained snails individually at 8–10 °C to prevent mortality resulting from reinfection (Kuris and Warren, 1980). This entire procedure took place in September 2007 and again in February 2008, resulting in a total of 27 infected snails.

#### 2.3. Amphibian collection and maintenance

On February 22, 2008, we collected four freshly laid *L. sylvaticus* egg masses from an ephemeral pond in Montgomery County, Virginia. We transferred egg masses gradually from pond water to a 3:1 mix of dechloraminated (ChlorAm-X®, AquaScience Research Group, Inc., North Kansas City, MO, USA) tap water (53.7 mg/L CaCO₃) and well water (364 mg/L), to create a mixture with an acceptable hardness level of 108 mg/L of CaCO₃. We removed sixty healthy *L. sylvaticus* eggs with intact jelly coats from each egg mass (240 total eggs) and acclimated them together in a single bin containing 6 L of water. We maintained the eggs at 18 °C using a temperature-controlled environmental chamber (Adapitis, Conviron, Manitoba, Canada.). All eggs hatched on March 2, and 80 tadpoles were selected randomly for the experiment.
and assigned to individual 4-L containers filled with 3 L of water. Prior to the experimental procedures, tadpoles were fed ad lib with a 3:1 mixture of ground rabbit chow and Tetra-Min® Flake Fish food.

2.4. Experimental design

We designed a regression-based laboratory experiment to investigate the energetic costs of *E. trivolvis* infection in tadpoles during late larval development and metamorphic climax because it is a more powerful approach than ANOVA for a given sample size of experimental units (Cottingham et al., 2005). We exposed tadpoles to cercariae gradually, rather than in a single pulse exposure, because this more closely approximates transmission that might occur in nature and also reduces mortality after initial encystment (Ballabeni and Ward, 1993; Torchin et al., 2005; Orlofske et al., 2013).

We randomly assigned individual tadpoles to one of eight treatments (N = 10 tadpoles/treatment) receiving a total of 0 (control), 15, 27, 45, 108, 135, 180 or 225 *E. trivolvis* cercariae. We exposed tadpoles to one-third of the total number of cercariae at each of three time points (19, 29, and 39 days post-hatch). At each time point, we stimulated six snails to shed cercariae under a heat lamp and pooled cercariae from at least 3 snails for each tadpole. We counted cercariae using a dissecting microscope, collected them with a glass pipette, and dispensed them into a 120-mL cup containing the tadpole in 40 mL of water. The average wet mass + 1 SE of the tadpole was 320 ± 9 mg (N = 80), 504 ± 16 mg (N = 79), and 710 ± 21 mg (N = 78) at the first, second, and third cercariae exposures, respectively. The ranges of developmental stages (Gosner, 1960) were 26–29, 27–33, and 28–37 at the three exposures, respectively.

We examined every individual at several time points throughout the experiment. First, immediately after each exposure, we monitored tadpoles for edema before being weighed and then returned them to their individual container. Tadpoles that exhibited edema were monitored every 12 h until recovery or death. Throughout the remainder of the experiment, we monitored tadpole mortality daily and tadpole mass weekly. We weighed tadpoles to the nearest 0.1 mg by removing the tadpole from the container with a net and blotting it with tissue paper to remove excess moisture. These measurements allowed us to assess growth rate and to calculate rations equal to 8% of each individual's body mass per day until the next measurement. We provided the rations three times a week after 50% water changes.

We examined all tadpoles with well-developed hind limbs for the presence of metatarsal tubercles but absence of visible front limbs (developmental stages 38–40, Gosner, 1960) with a dissecting microscope. After tadpoles reached this range of stages, we randomly selected 32 (N = 4 per treatment) for respiration and encystment measurements during late developmental stages (stages 38–40), while we allowed the remaining 48 tadpoles to complete metamorphosis (stage 46). For the 48 tadpoles raised through metamorphosis, we recorded the duration of larval development and mass at both stages 38–40 and 42. When these remaining tadpoles reached metamorphic climax (stage 42; determined by the emergence of at least one front limb) we began monitoring the duration of metamorphosis (in hours from stages 42 to 46), respiration, and loss of body mass during metamorphosis. Final mass was recorded for all individuals that reached stage 46 (N = 43).

2.5. Respirometry and encystment

We quantified oxygen consumption rates (O₂ mL/h) of tadpoles during late development (stages 38–40) and metamorphosis (stages 42–46). We used a general procedure and the same equipment for all respirometry measurements (described here) with some slight modifications based on life stage (described below). First, we used a computer-controlled, indirect, closed-circuit respirometer (Micro-Oxymax, Columbus Instruments, Columbus, OH, USA) with techniques similar to those used for pickerel frog (*L. palustris*) and wood frog tadpoles at earlier developmental stages (Orlofske et al., 2009; Orlofske and Hopkins, 2009; Orlofske et al., 2013). We used 100-mL sealed glass culture bottles as respirometry chambers. We recorded wet mass of individuals as described above, before placement in the respirometry chambers. We placed individuals in an environmental cabinet maintained at 18 °C during respirometry measurements. We calibrated the respirometer prior to each trial using a certified gas mixture. For quality assurance, we monitored oxygen consumption rates (mL/h) simultaneously in one control chamber containing a medical battery (Dura-Cell Procell Zinc Air Medical DA 146, 8.4 Volts) with a known rate of O₂ consumption, and one chamber filled only with water. Each air sample was dried using a hygrosopic drier containing nafion tubing (Columbus Instruments, Columbus, OH, USA) and adjusted for carbon dioxide (measured concurrently) prior to measuring tadpole respiration rates. Oxygen consumption was measured every 66 min and was corrected for standard temperature and pressure. Normoxic conditions were maintained by completely refreshing the air within the chamber headspace every 2.5 h. Each trial started at approximately the same time (1100–1200 h) to control for the influence of natural circadian rhythms on respiration (Roe et al., 2004b).

For respirometry of late developmental stages 38–40, we fasted individuals for 48 h prior to measurements to reduce metabolic contributions from digestion (Crowder et al., 1998). We filled respirometry chambers with 80 mL of well oxygenated, dechloraminated tap water. Each respirometry trial lasted 24 h after which we removed tadpoles from the chambers, and recorded stage, and mass to the nearest 0.1 mg. Because of the limited number of respirometry chambers, we completed respiration measurements of 22 tadpoles (N = 2–3/treatment group).

For respirometry trials during metamorphic climax (stages 42–46) fasting was not required because during metamorphosis tadpoles cease feeding while the mouthparts and digestive tract undergo substantial remodeling (Duellman and Trueb, 1986). We filled each chamber with 6 mL of well oxygenated, dechloraminated tap water to keep the metamorphosing individual hydrated, without drowning. We placed a 3.8 cm × 3.8 cm piece of plastic mesh against the side of each respirometry chamber, forming an inclined plane for emergence from the water that could facilitate air breathing using methods similar to Beck and Congdon (2003) and Orlofske and Hopkins (2009). We stopped and restarted respirometry trials every 24 h so that we could assess the developmental stage of the individual and refresh water in each chamber. After completing metamorphosis, we removed juveniles from the chambers and recorded wet mass to the nearest 0.1 mg. Similarly, we monitored development of the remaining individuals not used in respirometry trials every 12 h and recorded wet mass of after completion of metamorphosis. Respirometry measurements continued for each individual until completion of metamorphosis, indicated by complete tail resorption (stage 46). Again, based on the individual timing of metamorphosis and the limited numbers of chambers, we completed respirometry measurements for the entire duration of metamorphosis for a total of 28 individuals (N = 1–5/treatment group).

After respirometry measurements, we euthanized all individuals with MS-222 (tricaine methanesulfonate, ACROS Organics, Morris Plains, New Jersey). During dissections we removed and examined the pronephros, mesonephros, and connecting Wolffian ducts from each tadpole. For metamorphs, we examined the mesonephros, and tissue in the area surrounding the location of pronephros prior to degradation during metamorphic climax. Encysted *E. trivolvis* metacercariae were counted using a compound microscope.

2.6. Energy metabolism calculations

Prior to statistical analysis, we plotted O₂ consumption of each tadpole over time and visually assessed activity peaks because spontaneous activity can bias estimates of standard metabolic rate (SMR). Based on examination of the plots, we discarded the first measurement of each
sampling trial because it was often inflated by stress caused by handling before trials. To minimize the bias of tadpole activity on estimates of SMR (mL/h), we used the lowest quartile value as an estimate of SMR for each individual (Hopkins et al., 2004a). Visual examination of the plots revealed that this method effectively represented baseline oxygen consumption of each animal in our study.

We consolidated data from all respirometry trials for each tadpole that completed metamorphosis in the respirometry chambers to generate a continuous respiration profile that covered the entire metamorphic period (5–9 d) for that individual (as described in Orlofske and Hopkins, 2009). During the daily break between respirometry trials (3–5 h), we assumed that O₂ consumption rate (mL/h) remained constant from the last measurement before the break until the first valid measurement on the following day. Total oxygen consumed (mL) during metamorphosis was calculated as the sum of O₂ consumption rates (mL/h) multiplied by the duration of metamorphic climax (h). Because respirometry trials could begin only every 24 h, we were unable to obtain respiration data for individuals immediately after their front limbs emerged. For all individuals, oxygen consumption between front limb emergence and the first respirometry measurement was estimated by the average rate of oxygen consumption of their first six valid measurements multiplied by the hours (range 1.2–23.1 h) that the tadpole possessed front limbs prior to starting the respirometry trial. This amount then was added to their remaining respiration profile. A computer malfunction interrupted data collection for nine tadpoles for 12 h; the oxygen consumption during the missing interval was calculated using the same procedure as that for the interval between daily trials. After calculating the amount of oxygen used to complete metamorphosis, data were converted to Joules (J) using a conversion factor of 18.8 J/mL O₂ (Schmidt-Nielsen, 1990). Total energy costs were divided into maintenance costs and developmental costs following the procedure described in Beck and Congdon (2003) and Orlofske and Hopkins (2009). Briefly, In-transformed late-stage tadpole SMR and mass were regressed to provide the values of the constants used in an integration to calculate maintenance costs over time (see above). Assumptions of the integration included a linear decrease in mass over the course of metamorphosis and an exponential relationship between mass and SMR. We obtained an estimate of developmental energy costs by subtracting maintenance costs from total energy costs.

2.7. Statistical analysis

Data were tested to determine whether the assumptions of parametric models were met and appropriate transformations were made prior to statistical analysis. The number of metacercariae recovered required log transformation and percent encystment required arcsine square root transformation. Final larval mass and mass at stage 46 required log transformations prior to analysis. We calculated mass-specific growth rate using the change in natural log transformed mass divided by the duration of developmental period to represent a proportional increase in body size on a daily basis (Sinervo and Adolph, 1989). Values for SMR and mass were log transformed because metabolism is a power function of mass (Chappell et al., 1996). Total oxygen consumption calculated during metamorphic climax was also log transformed. Fasted tadpole masses were used in all analyses involving tadpole mass. All statistical tests were conducted using JMP 8.0 (SAS Institute, Cary, NC, USA). Statistical significance was assessed at α = 0.05.

Our sampling design allowed us to address the question of how parasite infection influenced growth and development during three developmental windows, Gosner stages 38–40 (late development), 42 (emergence of front limbs) and 46 (completion of metamorphosis). First, for the tadpoles measured at late development (stages 38–40), we performed three linear regressions with the number of metacercariae recovered from each tadpole as the independent variable and growth rate (mg/day), final mass (mg) and duration of development (days) as the three response variables.

Second, to test the effect of parasite infection on growth and duration of development of tadpoles measured at stage 42, it was first necessary to determine whether metacercariae were lost during metamorphic climax, because metacercariae were quantified at stage 46 for these individuals. Metacercariae frequently encyst in the pronephros, which is degraded during tadpole metamorphosis (Schottoefer et al., 2003; Belden, 2006), creating the possibility that our metacercarial counts at stage 46 may under estimate actual infections at stage 42. To determine if tadpoles sampled prior to metamorphic climax had higher infections than those sampled after metamorphosis, the number and percent of metacercariae recovered from tadpoles were compared between the two sampling time points where we quantified infections (Gosner 38–40 and Gosner 46) using ANCOVA with the number of cercariae to which tadpoles were exposed as the covariate in the model. We found that metacercarial infection intensity did not differ significantly between stages 38–40 and 46 (see Section 3.2) suggesting that infections were stable through development and that metacercariae were retained through metamorphosis. Therefore, it was appropriate to use the number of metacercariae recovered from animals after completing metamorphosis (stage 46) in a retrospective series of regression analyses examining growth rate (mg/day), final mass (mg) and duration of development (days) for the same tadpoles immediately prior to metamorphosis (stage 42).

Last, we conducted a series of analyses to examine the relationship between metacercariae and factors related specifically to metamorphic climax for tadpoles sampled at stage 46. To examine the relationship between the number of metacercariae and the duration of metamorphic climax (h), we used multiple linear regression with mass at stage 42 and the number of metacercariae as independent variables. We also used multiple linear regression to examine the influence of the number of metacercariae, mass at stage 42, and duration of climax on mass (mg) at the completion of metamorphic climax (stage 46). We were able to include both metacercariae and mass at stage 42 because these two variables were not significantly related to one another (see Section 3.3). Finally, we used multiple linear regressions to describe the relationship between the number of metacercariae and mass at stage 42 (independent variables) and the change and percent change in mass during climax (response variables).

To investigate the relationship between parasite infection and amphibian metabolism at late development (stages 38–40) and during metamorphosis (stages 42–46), we performed a series of multiple linear regressions. First, we used a multiple linear regression with metacercariae and body mass as independent variables and late stage tadpole SMR as the response variable to examine the role of parasites on host metabolism. To estimate the maintenance energy costs of tadpoles undergoing metamorphic climax, the coefficients of the regression of late stage tadpole ln transformed SMR and mass were used. Because metacercarial infection intensity did not significantly influence SMR (see Section 3.5), only mass was included in this second model to generate the values for metamorphic climax. The allometric equation is ln(SMR) = a + b ln (m), where SMR is the rate of oxygen consumption in mL/h, m is mass (g) and a and b are coefficients determined from the regression analysis. For tadpoles completing metamorphosis, total energy costs (O₂ mL), developmental energy costs, and percent of energy costs allocated to development were analyzed using multiple linear regression with both body mass and number of metacercariae as independent variables.

3. Results

3.1. Mortality and pathology post-infection

After the first exposure, 18 (22.5%) tadpoles exposed to 9–75 cercariae exhibited edema, which lasted 48–180 h with an average of 85.3 ± 40.3 (SD) h (N = 18). None of the tadpoles exhibited edema following the second and third exposures. Across the whole study, we observed...
low mortality (N = 7/80; 8.7%) that was spread across the seven treatment groups and three exposure periods. One tadpole exposed to 108 cercariae exhibited unusually arrested development (Gosner stage 38 for 3 weeks after all other tadpoles metamorphosed) and was excluded from all statistical analyses.

3.2. Encystment

After completing metamorphosis (Gosner 46), metacercariae were recovered from metamorphs in their mesonephros and in the region of the degenerated pronephros. There was no statistically significant difference in the number of encysted metacercariae between tadpoles sampled prior to or after completing metamorphosis (time of sampling p = 0.149, time x number of cercariae p = 0.352). The number of metacercariae recovered from all tadpoles and metamorphs combined was positively related to the number of cercariae to which they were exposed (R² = 0.71, p < 0.0001). The average number of metacercariae in the highest exposure group (exposed to 225 cercariae) was 59.7 ± 7.8 (SD) compared to 4.0 ± 1.6 (SE) in the lowest exposure group (exposed to 15 cercariae). However, the percentage of cercariae recovered as metacercariae was not related to the number of cercariae exposed (p = 0.510) or time of sampling (p = 0.075; time x number of cercariae p = 0.068; Table 1); across all parasite exposures an average of 27.3 ± 15.14 (SD) % of cercariae successfully encysted.

3.3. Growth and development

Larval mass of tadpoles at stages 38–40 (late stage) averaged 917 ± 140 (SD) mg (N = 29) and the larval period to this stage averaged 44.5 ± 10.0 (SD) d. Mass specific growth rate had a negative but non-significant correlation with number of metacercariae recovered post-mortem (R² = 0.11, p = 0.081, Fig. 1A). Furthermore, larval mass at stages 38–40 (R² = 0.02, p = 0.392) was not significantly correlated with the metacercariae intensity. The duration of the larval period to this stage was positively correlated to the number of metacercariae (R² = 0.33, p = 0.001, Fig. 1B), with each metacercaria adding ~0.25 day to development.

Tadpoles weighed immediately prior to metamorphosis (stage 42) averaged 979 ± 172 (SD) mg (N = 43) and the duration of the larval period to this stage averaged 53.7 ± 5.7 (SD) d. Mass-specific growth rate (R² = 0.00, p = 0.849, Fig. 1A) and final larval mass (R² = 0.00, p = 0.894) were not significantly correlated with the number of metacercariae. Similarly, there was no significant relationship between developmental period to stage 42 and number of metacercariae (R² = 0.06, p = 0.127, Fig. 1B).

3.4. Metamorphosis

The duration of metamorphic climax varied widely (Table 2) and was positively correlated to tadpole mass at the initiation of climax (p < 0.001), but not to the number of metacercariae recovered postmortem (p = 0.611). The final mass of tadpoles at stage 46 was positively correlated to mass at initiation of climax (p < 0.001), marginally negatively correlated to the duration of climax (p = 0.057), and not related to the number of metacercariae (p = 0.573). Tadpoles lost approximately one-third of their total mass during metamorphosis (Table 2). Mass loss showed a positive correlation with tadpole mass at initiation of climax (p < 0.001) and a marginally significant positive correlation with duration of climax (p = 0.058), but no relationship to the number of metacercariae (p = 0.821). The percentage of mass lost during metamorphosis was not related to either the number of metacercariae recovered postmortem (p = 0.620), or initial mass

| Table 1 |
|-----------------|--------|---|---|---|
| Percent encystment of Echinostoma trivolvis metacercariae in Lithobates sylvaticus tadpoles measured at late development (stages 38–40, Gosner, 1960) and after metamorphic climax (stage 46) after gradual exposure to a range of cercariae (range of final exposure: 15–225) exposures in the laboratory occurring 19, 29, and 39 d post-hatch. | | | |
| **Variable** | **N** | **Median** (SD) | **Minimum** (SD) | **Maximum** (SD) |
| Time | | | | |
| Late stage | 25 | 30.77 16.9 | 8.88 | 77.77 |
| Metamorphs | 37 | 24.34 15.0 | 0.00 | 60.00 |
| *a* Excluding controls. | | | | |
(p = 0.391), but was positively correlated to the duration of metamorphic climax (p = 0.033, Fig. 2A).

3.5. Energetics

Late-stage (Gosner 38–40) tadpoles used for estimation of SMR had an average mass of 918 ± 150 (SD) mg (N = 22). The average SMR of all late stage tadpoles was 0.088 ± 0.018 (SD) mL O₂/h. The number of metacercariae encysted did not significantly affect SMR (p = 0.437), but SMR was positively related to tadpole mass (p = 0.008). To obtain the constants for the calculation of maintenance energy for metamorphic climax, we also generated a second simplified regression model including only ln-transformed mass and SMR (because encystment was non-significant in the full model) of the late-stage tadpoles that showed a significant positive correlation (R² = 0.30, p = 0.009). The equation approximating this relationship was: ln(SMR) = 3.3571 + 1.4149 ln(m).

During metamorphic climax, tadpoles maintained an average metabolic rate of 0.130 ± 0.024 (SD) mL/h (N = 28), which resulted in an average total of 20.10 ± 6.12 (SD) mL O₂ consumed (Table 2). The metabolic rate was variable during climax, but no trends corresponded to time or any particular developmental stages. Instead, cumulative oxygen consumption increased linearly. The number of metacercariae did not significantly affect total mL of O₂ consumed during metamorphosis (p = 0.278). However, both initial mass (p < 0.0001) and duration of climax (p < 0.0001) were positively correlated with total mL O₂ consumed. Lithobates sylvaticus tadpoles required an average of 377.83 J of energy to complete the metamorphic transition, which was allocated into approximately 26% maintenance and 74% developmental energy (Table 2). The amount of energy allocated to development was positively correlated with tadpole mass at the initiation of metamorphosis (p < 0.0001) and with the duration of climax (p < 0.0001, Fig. 2B), but not related to the number of metacercariae (p = 0.654). The percentage of energy allocated to development was not correlated with the number of metacercariae (p = 0.945), initial mass (p = 0.084), or duration of climax (p = 0.189).

4. Discussion

Using a laboratory experiment to gradually expose tadpoles to a realistic range of infection intensities, we found that *E. trivolvis* metacercariae had a negative, but not statistically significant affect, on mass-specific growth rate. In addition, exposure led to a significantly longer period of development to stages 38–40. However, no significant effects of infection were observed during metamorphosis, supporting the idea that parasite effects are host-stage specific (Holland et al., 2007). While our results indicate that amphibian metamorphosis is a critical transition period with significant energetic costs, concurrent infection with *Echinostoma trivolvis* trematode metacercariae did not significantly alter these energetic costs. In comparison to other species, the energetic costs of metamorphic climax in *Lithobates sylvaticus* correspond with costs reported for other ranid species, but may differ from *Anaxyrus terrestris*, which has a different life history strategy (Beck and Congdon, 2003). While most research addressing energetic trade-offs between parasite defense and host processes has focused on a narrow range of standardized conditions, our work contributes to the integration of physiology and ecoimmunology by considering parasite infection with simultaneous energetic demands of stage-specific developmental processes (Robar et al., 2011; Warne et al., 2011).

Duration of development for late stage tadpoles was negatively correlated with *E. trivolvis* infection intensity, extending the range of stages negatively impacted by infection, either as the result of patholgy or a developmental response or recovery from previous pathology (Fried et al., 1997; Belden, 2006; Holland et al., 2007). The period of development immediately prior to metamorphic climax, stages 39–41, is an important life history stage for amphibians. Because tadpoles are particularly vulnerable to predation during metamorphic climax (Wassersug and Sperry, 1977; Arnold and Wassersug, 1978), there may be sufficient selection for synchronous metamorphosis to satiate predators as a survival mechanism (Arnold and Wassersug, 1978). Therefore, delayed initiation of metamorphosis may increase fitness costs due to predator-induced mortality of individuals completing metamorphosis later or increased risk of further parasite exposure (Raffel et al., 2010; Belden and Wojdak, 2011). Despite the negative effects on developmental time due to infection observed for late stage tadpoles, energetic costs were not influenced by the number of metacercariae, similar to our findings for tadpoles undergoing metamorphosis and in a previous study on *L. palustris* tadpoles (Orlofske et al., 2009).

We predicted that parasite infection would increase energy use and delay development during metamorphosis, based on the significant contributions kidneys make to standard metabolic demands coupled with energetic costs of conditions like development (Steyermark et al., 2005; de Souza and Kuribara, 2006; Robar et al., 2011). In our study, metacercariae may not have increased host metabolic rates during metamorphosis if their presence does not interfere significantly with pronephros degradation (Fox, 1963). The maturing mesonephros may have compensated for any interference of kidney function or the biased distribution of metacercariae between kidneys may have reduced energy costs by localizing damage (Johnson et al., 2014). Furthermore, energetic costs of infection may only be apparent during metacercariae development (Lemly and Esch, 1984); however, in *L. palustris* tadpoles earlier in development, an energetic response was not detected during encystment of *E. trivolvis* (Orlofske et al., 2009). Building on this earlier study, we found that *E. trivolvis* metacercariae did not influence energetics of metamorphosis, the duration of metamorphic climax, final mass, change in mass, and percentage of initial mass lost during climax. If energetic costs of infection are related to immune function, the

![Fig. 2. A. Regression of duration of climax (h) and the percent change in mass of tadpoles completing metamorphic climax (p < 0.0001, N = 43). B. Regression of the duration of climax (h) and energy costs of development (J) (p = 0.033, N = 43).](image-url)
suppression of the immune response during amphibian metamorphosis related to loss and reorganization of tissues, as well as destruction of lymphocytes, could help explain the lack of observed energetic costs (Rollins-Smith, 1998). Parasites, such as the trematodes Clonostomum sp. and Ribeirioa ondatrae, with different body sizes and pathological impacts, might be expected to have more significant energetic or developmental costs prior to and throughout metamorphosis and would be useful models for future investigations of energetic costs of parasitism in larval amphibian hosts (Blaustein et al., 2012; Koprivnikar et al., 2012).

By examining consequences of infection at two stages of host development, our research also assessed how pathology and parasite infection changes over time. After the initial exposure to cercariae, 28% and 19% of the tadpoles exhibited edema in the late developmental stage and metamorphic climax. Both melanized cysts, occasionally surrounded by a fibrous capsule of host-derived tissue, and viable cysts were recovered from both late developmental stage tadpoles and metamorphs (Martin and Conn, 1990). The number of metacercariae recovered from both late developmental stage tadpoles and metamorphs was positively related to the total cercariae exposure. The slightly lower average percent metacercariae recovered after metamorphosis could be attributed to a longer time available for host immune responses to degrade cysts or a loss of cysts during the degradation of the pronephros during metamorphosis (Fox, 1963; Belden, 2006). Unmelanized, and potentially viable metacercariae were observed in mesonephros and the location of the degraded pronephros in metamorphs, supporting the conclusion that some cysts can survive the degradation of pronephros during metamorphosis (Fried et al., 1997; Thiessmann and Wassersug, 2000a; Schotttoef er et al., 2003). This is in contrast to earlier studies where cysts were not recovered in the region of the pronephros postmetamorphosis (Belden, 2006).

Importantly, quantification of the energetic costs of amphibian metamorphosis contributes to our ability to compare costs across species and amphibian life history strategies. The total energetic costs of metamorphosis in *L. sylvaticus* were 377.8 J at 18 °C in comparison to 424.5 J for *L. palustris* at 25 °C (Orlofske and Hopkins, 2009), 904 J for *Hoplobatrachus tigerinus* at 27 °C (Pandian and Marian, 1985), and 50.7 J for *Anaxyrus terrestris* at 25 °C (Beck and Congdon, 2003). Duration of climax and tadpole mass differed greatly among studies; however, qualitative comparisons using isometric relationships with mass and time (i.e., total energy use converted to 1/g/h) can be informative. This comparison yields very similar energy expenditure for *L. sylvaticus* (2.53 J/g/h) in comparison to *L. palustris* (2.57 J/g/h) and *H. tigerinus* (2.63 J/g/h), the other members of the family Ranidae, which all differ from the toad *A. terrestis* (6.76 J/g/h). The amount of energy allocated to development for *L. sylvaticus* was approximately 74%, which is higher than both *L. palustris* and *A. terrestis*, which required 50% and 40% respectively (Beck and Congdon, 2003; Orlofske and Hopkins, 2009). Although total energetic costs are higher, large tadpoles complete metamorphosis more efficiently by using proportionally less total energy for climax than small tadpoles. However, in contrast to *L. palustris* and *A. terrestis* (Beck and Congdon, 2003; Orlofske and Hopkins, 2009), the negative relationship between percent development costs and mass was not significant in *L. sylvaticus*, suggesting the efficiency associated with development at a larger body size was not as pronounced. The temperature used for measurements of *L. sylvaticus* may not have been the most efficient temperature for development and could have contributed to the relatively long duration of climax, which was longer than the other Ranid species despite the smaller body size of *L. sylvaticus*. The duration of climax contributed significantly to the total energy and developmental energy expended, which further supports the conclusion that more slowly developing tadpoles require more energy for metamorphosis (Orlofske and Hopkins, 2009).

Our study characterized developmental components associated with metamorphosis that may influence fitness. Interactions among duration of climax, initial mass, and final mass indicated that initial larval size significantly affects the length of metamorphic climax, change in mass, and the final metamorphic size. The duration of climax also influences final size, and the amount and percentage of mass lost. The size advantage large tadpoles maintained after completing metamorphosis may increase fitness through higher juvenile survival, reduced time to maturity, and increased fecundity (Semlitsch et al., 1988; Berven, 1988, 1990; Semlitsch and Gibbons, 1990; Scott, 1994; Beck and Congdon, 1999; Beck and Congdon, 2000; Boone and Bridges, 2003; Orlofske et al., 2009; Todd et al., 2011, 2012). Therefore, developmental effects at early life history stages may have legacy effects for adult reproduction.

Overall, our research contributes to our knowledge of the physiological costs of parasitism concurrently with other demands, an important component of the ecomunimunity framework in disease ecology (Hawley and Altizer, 2010). While energetically costly, amphibian metamorphosis appeared to be unaffected by parasites acquired during aquatic larval stages. However, parasitism negatively affected time to developmental stages immediately prior to metamorphosis, suggesting that parasites may contribute to differential impacts depending on host age. Environmental influences must be accounted for when examining the effects of parasites on amphibian metamorphosis. For amphibians that breed in temporary or semi-permanent wetlands, metamorphosis often coincides with resource limitation and pond drying, conditions where the effects of parasite infection may be more detrimental (Kiesecker and Skelly, 2001; Koprivnikar et al., 2014). Additional physiological and biochemical studies are needed to help clarify the mechanisms of how macroparasites, including *E. trivolvis*, affect their amphibian hosts and the potential interaction with environmental factors (Warne et al., 2011; Koprivnikar et al., 2012).

Acknowledgments

This study was supported by the Department of Fish and Wildlife Conservation at Virginia Tech, a National Science Foundation (IOB 0615361) to WAH, and a NSF Graduate Research Fellowship to SAO (DGE 0707432). A Grant-In-Aid-Of-Research from Sigma Xi to SAO provided additional support. R.C. Jadin provided assistance in collecting amphibian eggs and maintaining the metamorphs. The manuscript was greatly improved by comments from J.T. Hoverman, P.D. Widder, J.M. Orlofske, C. Orlofske, and R.C. Jadin and two anonymous reviewers. This research was approved by the Virginia Tech Institutional Animal Care and Use Committee (07-021-FIW).

References

Beaver, P.C., 1937. Experimental studies on Echinostoma revolutum (Froelich), a fluke from birds and mammals III. Biol. Monogr. 15, 1–96.


