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

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4

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26 **Abstract**

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

48 processes by considering parasite infection with simultaneous energetic demands during a  
49 sensitive period of development.

50

51 **Key words:** energy, parasite, tadpole, trematode, oxygen consumption, respiration,  
52 metamorphic climax, metabolism

53

## 54 **1. Introduction**

55

56 Fundamental to understanding animal physiology is the concept of energetic trade-offs  
57 among the competing processes of growth, development, maintenance and reproduction  
58 (Stearns, 1989; Roff, 2001; Zera and Harshman, 2001; Lee, 2006). Amongst the  
59 multitude of physiological costs inherent to self-maintenance, immune defense against  
60 parasite infection is thought to be particularly costly (Martin et al., 2003; Lee, 2006;  
61 Hawley and Altizer, 2010). For example, basal metabolic rate of Collared Doves  
62 (*Streptopelia decaocto*) increased by a maximum of 8.5% in response to challenge by a  
63 novel antigen, corresponding with antibody production (Eraud et al., 2005). However,  
64 there are relatively few studies quantifying the metabolic costs of immune challenge in  
65 wild vertebrate species, especially in response to parasites (Lochmiller and Deerenberg,  
66 2000; Hawley et al., 2012). Costs of parasite defense are not limited solely to support of  
67 the immune system. They can also consist of repairing tissue damage, and can result from  
68 competition between the parasite and host for energy resources (Kristan and Hammond,  
69 2000; Khokhlova et al., 2002; Sandland and Minchella, 2003).

70 Defense against parasites may elicit trade-offs with other functions or activities  
71 that require common resources, thereby influencing an animal's fitness (Lee, 2006;  
72 Hawley and Altizer, 2010). For example, wood frog tadpoles exposed to ranavirus  
73 showed elevated corticosterone, which was associated with a more rapid progression  
74 through metamorphosis at the expense of body weight and immune responses (Warne et  
75 al., 2011). This illustrates how intrinsic factors, such as particular developmental periods,  
76 require increased energy allocation. This can potentially limit the investment in other  
77 processes, such as immunity, and lead to increased fitness-related consequences of  
78 infection (Warne et al., 2011, Blaustein et al., 2012). Using this resource allocation  
79 framework helps explain why there may not be trade-offs between parasite defense and  
80 other demands unless they share required resources, occur simultaneously, or if available  
81 resources are insufficient to fuel competing demands (Lee, 2006; Hawley et al., 2012).  
82 Across a variety of host-parasite systems energetic costs of parasite infection were only  
83 evident or additive when there were competing energetic demands, such as during  
84 maximum activity, temperature stress, or during mammalian pregnancy or lactation  
85 (Lester, 1971; Meakins and Walkey, 1975; Hayworth et al., 1987; Munger and Karasov,  
86 1989; Connors and Nickol, 1991; Booth et al., 1993; Chappell et al., 1996; Meagher and  
87 O'Connor, 2001; Kristan and Hammond, 2000, 2003; Hawley et al., 2012; Novikov et al.,  
88 2015). Therefore, it is important to investigate energetic costs of parasitism during  
89 periods of elevated energy demand to determine the overall impact of parasites on hosts  
90 (Robar et al., 2011; Warne et al., 2011).

91 Larval amphibians and trematode parasites have become a model system for  
92 investigating many aspects of host-parasite interactions and could be used specifically to

93 test physiological trade-offs of parasite defense and development (Warne et al., 2011;  
94 Blaustein et al., 2012; Koprivnikar et al., 2012). *Echinostoma trivolvis* is a widespread  
95 digenetic trematode infecting larvae of several amphibian species as intermediate hosts  
96 and occasionally causing mortality and reduced growth, especially in very small larvae  
97 (Beaver, 1937; Fried et al., 1997; Schotthoefer et al., 2003; Belden, 2006; Holland et al.,  
98 2007; Belden and Wojdak, 2011). Specifically, *E. trivolvis* metacercariae infect  
99 amphibian kidneys, causing renal inflammation, which can result in physiological  
100 dysfunction and edema (McClure, 1919; Faeh et al., 1998). Little is known about the  
101 immune response of larval amphibians to helminths, such as trematodes (Holland 2009,  
102 Koprivnikar et al. 2012). However, *E. trivolvis* infection in amphibians is associated with  
103 granuloma formation, granulocyte infiltration, and a shift in the abundance and types of  
104 circulating leukocytes (Martin and Conn 1990; Holland et al. 2007). Although some  
105 previous investigations of *E. trivolvis* infection in larval amphibians revealed reductions  
106 in growth with likely energetic underpinnings, no significant effects on host metabolic  
107 rate have been detected (Fried et al., 1997; Schotthoefer et al., 2003; Orlofske et al.,  
108 2009, 2013). However, it is possible that effects to host metabolism may become evident  
109 during developmental periods that are more demanding, such as amphibian  
110 metamorphosis (Warne et al., 2011; Blaustein et al., 2012).

111         Studies of amphibian metamorphosis indicate that this is an energetically  
112 demanding period where total energetic costs and developmental costs are significant  
113 (*Hoplobatrachus* [=*Rana*] *tigerinus* Pandian and Marian, 1985; *Anaxyrus* [=*Bufo*]  
114 *terrestris*, Beck and Congdon, 2003; and *Lithobates palustris*, Orlofske and Hopkins,  
115 2009). Compensatory responses of hosts to parasites could be limited during

116 metamorphosis because of reliance upon stored energy resources (Duellman and Trueb,  
117 1986; Beck and Congdon, 2003) and the potential ecological vulnerabilities imposed by  
118 delayed metamorphosis (Wassersug and Sperry, 1977; Arnold and Wassersug, 1978;  
119 Downie et al., 2004).

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

135

## 136 **2. Materials and Methods**

### 137 *2.1. Study system*

138 *Echinostoma trivolvis* is a model parasite used frequently to investigate host-parasite  
139 interactions (Thiemann and Wassersug, 2000a,b; Belden, 2006; Koprivnikar et al., 2006;  
140 Toledo et al., 2007; Griggs and Belden, 2008; Johnson and McKenzie, 2008).

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

149 Wood frogs (*Lithobates* [= *Rana*] *sylvaticus*) are the most broadly distributed  
150 amphibian in North America (Redmer and Trauth, 2005) and are host to a diversity of  
151 adult and larval parasites (McAllister et al., 1995). One of the most commonly  
152 documented trematodes of wild *L. sylvaticus* tadpoles is *Echinostoma trivolvis* (Najarian,  
153 1955; McAllister et al., 1995; Woodhams et al., 2000). In *L. sylvaticus*, natural infections  
154 with echinostomes averaged 90 metacercariae per host (Woodhams et al., 2000).

155

## 156 2.2. Parasite culture

157 Methods for obtaining infected snails follow Orlofske et al. (2013). Briefly, *Echinostoma*  
158 *trivolvis* eggs were collected by mixing feces from laboratory- infected golden hamsters  
159 (*Mesocricetus auratus*) with a small amount of water, and adding it to containers with  
160 laboratory-raised *Planorbella trivolvis* snails. We did not quantify the number of eggs in



161 the feces dilution, but similar collections from the same hamsters yielded 666–1043  
162 eggs/mL. Water in the snail containers was left undisturbed for 3 weeks to allow for  
163 hatching of *E. trivolvis* eggs (Belden et al., 2009). We maintained snails for 3 weeks at  
164 room temperature with lettuce and flake fish food provided *ad lib* and 50% water changes  
165 performed weekly. We screened snails for infection by placing them in individual  
166 containers warmed with an incandescent bulb and microscopically examined the water  
167 for cercariae (Schmidt and Fried, 1996). After we confirmed parasite infection, we  
168 maintained snails individually at 8–10°C to prevent mortality resulting from reinfection  
169 (Kuris and Warren, 1980). This entire procedure took place in September 2007 and again  
170 in February 2008, resulting in a total of 27 infected snails.

171

### 172 2.3. Amphibian collection and maintenance

173 On February 22, 2008, we collected four freshly laid *L. sylvaticus* egg masses from an  
174 ephemeral pond in Montgomery County, Virginia. We transferred egg masses gradually  
175 from pond water to a 3:1 mix of dechloraminated (ChlorAm-X<sup>®</sup>, AquaScience Research  
176 Group, Inc., North Kansas City, MO, USA) tap water (53.7 mg/L CaCO<sub>3</sub>) and well water  
177 (364 mg/L), to create a mixture with an acceptable hardness level of 108 mg/L of CaCO<sub>3</sub>.  
178 We removed sixty healthy *L. sylvaticus* eggs with intact jelly coats from each egg mass  
179 (240 total eggs) and acclimated them together in a single bin containing 6 L of water. We  
180 maintained the eggs at 18°C using a temperature-controlled environmental chamber  
181 (Adaptis, Conviron, Manitoba, Canada). All eggs hatched on March 2, and 80 tadpoles  
182 were selected randomly for the experiment and assigned to individual 4-L containers

183 filled with 3 L of water. Prior to the experimental procedures, tadpoles were fed *ad lib*  
184 with a 3:1 mixture of ground rabbit chow and Tetra-Min® Flake Fish food.

185

#### 186 2.4. Experimental design

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

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

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

215           We examined all tadpoles with well-developed hind limbs for the presence of  
216 metatarsal tubercles but absence of visible front limbs (developmental stages 38–40,  
217 Gosner, 1960) with a dissecting microscope. After tadpoles reached this range of stages,  
218 we randomly selected 32 (N = 4 per treatment) for respiration and encystment  
219 measurements during late developmental stages (stage 38–40), while we allowed the  
220 remaining 48 tadpoles to complete metamorphosis (stage 46). For the 48 tadpoles raised  
221 through metamorphosis, we recorded the duration of larval development and mass at both  
222 stage 38–40 and 42. When these remaining tadpoles reached metamorphic climax (stage  
223 42; determined by the emergence of at least one front limb) we began monitoring the  
224 duration of metamorphosis (in hours from stage 42 to 46), respiration, and loss of body  
225 mass during metamorphosis. Final mass was recorded for all individuals that reached  
226 stage 46 (N = 43).

227

228 2.5. *Respirometry and encystment*

229 We quantified oxygen consumption rates ( $O_2$  ml/hr) of tadpoles during late development  
230 (stages 38–40) and metamorphosis (stage 42–46). We used a general procedure and the  
231 same equipment for all respirometry measurements (described here) with some slight  
232 modifications based on life stage (described below). First, we used a computer-  
233 controlled, indirect, closed-circuit respirometer (Micro-Oxymax, Columbus Instruments,  
234 Columbus, OH, USA) with techniques similar to those used for pickerel frog (*L.*  
235 *palustris*) and wood frog tadpoles at earlier developmental stages (Orlofske et al., 2009;  
236 Orlofske and Hopkins, 2009; Orlofske et al., 2013). We used 100-mL sealed glass culture  
237 bottles as respirometry chambers. We recorded wet mass of individuals as described  
238 above, before placement in the respirometry chambers. We placed individuals in an  
239 environmental cabinet maintained at 18°C during respirometry measurements. We  
240 calibrated the respirometer prior to each trial using a certified gas mixture. For quality  
241 assurance, we monitored oxygen consumption rates (mL/h) simultaneously in one control  
242 chamber containing a medical battery (Duracell Procell Zinc Air Medical DA 146, 8.4  
243 Volts) with a known rate of  $O_2$  consumption, and one chamber filled only with water.  
244 Each air sample was dried using a hygroscopic drier containing nafion tubing (Columbus  
245 Instruments, Columbus, OH USA) and adjusted for carbon dioxide (measured  
246 concurrently) prior to measuring tadpole respiration rates. Oxygen consumption was  
247 measured every 66 minutes and was corrected for standard temperature and pressure.  
248 Normoxic conditions were maintained by completely refreshing the air within the  
249 chamber headspace every 2.5 h. Each trial started at approximately the same time (1100–  
250 1200 h) to control for the influence of natural circadian rhythms on respiration (Roe et

251 al., 2004).

252 For respirometry of late developmental stages 38–40, we fasted individuals for 48  
253 h prior to measurements to reduce metabolic contributions from digestion (Crowder et al.,  
254 1998). We filled respirometry chambers with 80 ml of well oxygenated, dechloraminated  
255 tap water. Each respirometry trial lasted 24 h after which we removed tadpoles from the  
256 chambers, and recorded stage, and mass to the nearest 0.1 mg. Because of the limited  
257 number of respirometry chambers, we completed respiration measurements of 22  
258 tadpoles (N = 2–3/ treatment group).

259 For respirometry trials during metamorphic climax (stage 42–46) fasting was not  
260 required because during metamorphosis tadpoles cease feeding while the mouthparts and  
261 digestive tract undergo substantial remodeling (Duellman and Trueb, 1986). We filled  
262 each chamber with 6 mL of well oxygenated, dechloraminated tap water to keep the  
263 metamorphosing individual hydrated, without drowning. We placed a 3.8 cm x 3.8 cm  
264 piece of plastic mesh against the side of each respirometry chamber, forming an inclined  
265 plane for emergence from the water that could facilitate air breathing using methods  
266 similar to Beck and Congdon (2003) and Orlofske and Hopkins (2009). We stopped and  
267 restarted respirometry trails every 24 h so that we could assess the developmental stage of  
268 the individual and refresh water in each chamber. After completing metamorphosis, we  
269 removed juveniles from the chambers and recorded wet mass to the nearest 0.1 mg.  
270 Similarly, we monitored development of the remaining individuals not used in  
271 respirometry trials every 12 h and recorded wet mass of after completion of  
272 metamorphosis. Respirometry measurements continued for each individual until  
273 completion of metamorphosis, indicated by complete tail resorption (stage 46). Again,

274 based on the individual timing of metamorphosis and the limited numbers of chambers,  
275 we completed respirometry measurements for the entire duration of metamorphosis for a  
276 total of 28 individuals (N = 1–5/treatment group).

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

284

#### 285 *2.6. Energy metabolism calculations*

286 Prior to statistical analysis, we plotted O<sub>2</sub> consumption of each tadpole over time and  
287 visually assessed activity peaks because spontaneous activity can bias estimates of  
288 standard metabolic rate (SMR). Based on examination of the plots, we discarded the first  
289 measurement of each sampling trial because it was often inflated by stress caused by  
290 handling before trials. To minimize the bias of tadpole activity on estimates of SMR  
291 (mL/hr), we used the lowest quartile value as an estimate of SMR for each individual  
292 (Hopkins et al., 2004). Visual examination of the plots revealed that this method  
293 effectively represented baseline oxygen consumption of each animal in our study.

294         We consolidated data from all respirometry trials for each tadpole that completed  
295 metamorphosis in the respirometry chambers to generate a continuous respiration profile  
296 that covered the entire metamorphic period (5–9 d) for that individual (as described in

297 Orlofske and Hopkins, 2009). During the daily break between respirometry trials (3–5 h),  
298 we assumed that O<sub>2</sub> consumption rate (mL/h) remained constant from the last  
299 measurement before the break until the first valid measurement on the following day.  
300 Total oxygen consumed (mL) during metamorphosis was calculated as the sum of O<sub>2</sub>  
301 consumption rates (mL/h) multiplied by the duration of metamorphic climax (h). Because  
302 respirometry trials could begin only every 24 h, we were unable to obtain respiration data  
303 for individuals immediately after their front limbs emerged. For all individuals, oxygen  
304 consumption between front limb emergence and the first respirometry measurement was  
305 estimated by the average rate of oxygen consumption of their first six valid  
306 measurements multiplied by the hours (range 1.2–23.1 h) that the tadpole possessed front  
307 limbs prior to starting the respirometry trial. This amount then was added to their  
308 remaining respiration profile. A computer malfunction interrupted data collection for nine  
309 tadpoles for 12 h; the oxygen consumption during the missing interval was calculated  
310 using the same procedure as that for the interval between daily trials.

311         After calculating the amount of oxygen used to complete metamorphosis, data  
312 were converted to Joules (J) using a conversion factor of 18.8 J/mL O<sub>2</sub> (Schmidt-Nielsen,  
313 1990). Total energy costs were divided into maintenance costs and developmental costs  
314 following the procedure described in Beck and Congdon (2003) and Orlofske and  
315 Hopkins (2009). Briefly, ln-transformed late-stage tadpole SMR and mass were regressed  
316 to provide the values of the constants used in an integration to calculate maintenance  
317 costs over time (see above). Assumptions of the integration included a linear decrease in  
318 mass over the course of metamorphosis and an exponential relationship between mass

319 and SMR. We obtained an estimate of developmental energy costs by subtracting  
320 maintenance costs from total energy costs.

321

## 322 *2.7. Statistical analysis*

323 Data were tested to determine whether the assumptions of parametric models were met  
324 and appropriate transformations were made prior to statistical analysis. The number of  
325 metacercariae recovered required log transformation and percent encystment required  
326 arcsine square root transformation. Final larval mass and mass at stage 46 required log  
327 transformations prior to analysis. We calculated mass-specific growth rate using the  
328 change in natural log transformed mass divided by the duration of developmental period  
329 to represent a proportional increase in body size on a daily basis (Sinervo and Adolph  
330 1989). Values for SMR and mass were log transformed because metabolism is a power  
331 function of mass (Chappell et al., 1996). Total oxygen consumption calculated during  
332 metamorphic climax was also log transformed. Fasted tadpole masses were used in all  
333 analyses involving tadpole mass. All statistical tests were conducted using JMP 8.0 (SAS  
334 Institute, Cary, NC USA). Statistical significance was assessed at  $\alpha = 0.05$ .

335 Our sampling design allowed us to address the question of how parasite infection  
336 influenced growth and development during three developmental windows, Gosner stage  
337 38–40 (late development), 42 (emergence of front limbs) and 46 (completion of  
338 metamorphosis). First, for the tadpoles measured at late development (stage 38–40), we  
339 performed three linear regressions with the number of metacercariae recovered from each  
340 tadpole as the independent variable and growth rate (mg/day), final mass (mg) and  
341 duration of development (days) as the three response variables.



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

361           Last, we conducted a series of analyses to examine the relationship between  
362 metacercariae and factors related specifically to metamorphic climax for tadpoles  
363 sampled at stage 46. To examine the relationship between the number of metacercariae  
364 and the duration of metamorphic climax (h), we used multiple linear regression with mass

365 at stage 42 and the number of metacercariae as independent variables. We also used  
366 multiple linear regression to examine the influence of the number of metacercariae, mass  
367 at stage 42, and duration of climax on mass (mg) at the completion of metamorphic  
368 climax (stage 46). We were able to include both metacercariae and mass at stage 42  
369 because these two variables were not significantly related to one another (see *Results*  
370 3.3). Finally, we used multiple linear regressions to describe the relationship between the  
371 number of metacercariae and mass at stage 42 (independent variables) and the change  
372 and percent change in mass during climax (response variables).

373 To investigate the relationship between parasite infection and amphibian  
374 metabolism at late development (stage 38–40) and during metamorphosis (stage 42–46),  
375 we performed a series of multiple linear regressions. First, we used a multiple linear  
376 regression with metacercariae and body mass as independent variables and late stage  
377 tadpole SMR as the response variable to examine the role of parasites on host  
378 metabolism. To estimate the maintenance energy costs of tadpoles undergoing  
379 metamorphic climax, the coefficients of the regression of late stage tadpole  $\ln$   
380 transformed SMR and mass were used. Because metacercariae infection intensity did not  
381 significantly influence SMR (see Results), only mass was included in this second model  
382 to generate the values for metamorphic climax. The allometric equation is  $\ln(\text{SMR}) = a +$   
383  $b \ln(m)$ , where SMR is the rate of oxygen consumption in ml/h,  $m$  is mass (g) and  $a$  and  $b$   
384 are coefficients determined from the regression analysis. For tadpoles completing  
385 metamorphosis, total energy costs ( $\text{O}_2$  ml), developmental energy costs, and percent of  
386 energy costs allocated to development were analyzed using multiple linear regression  
387 with both body mass and number of metacercariae as independent variables.

388

### 389 **3. Results**

#### 390 *3.1. Mortality and pathology post-infection*

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

398

#### 399 *3.2. Encystment*

400 After completing metamorphosis (Gosner 46), metacercariae were recovered from  
401 metamorphs in their mesonephros and in the region of the degenerated pronephros. There  
402 was no statistically significant difference in the number of encysted metacercariae  
403 between tadpoles sampled prior to or after completing metamorphosis (time of sampling  
404  $p = 0.149$ , time X number of cercariae  $p = 0.352$ ). The number of metacercariae  
405 recovered from all tadpoles and metamorphs combined was positively related to the  
406 number of cercariae to which they were exposed ( $R^2 = 0.71$ ,  $p < 0.0001$ ). The average  
407 number of metacercariae in the highest exposure group (exposed to 225 cercariae) was  
408  $59.7 \pm 7.8$  (SE) compared to  $4.0 \pm 1.6$  (SE) in the lowest exposure group (exposed to 15  
409 cercariae). However, the percentage of cercariae recovered as metacercariae was not  
410 related to the number of cercariae exposed ( $p = 0.510$ ) or time of sampling ( $p = 0.075$ ;

411 time X number of cercariae  $p = 0.068$ ; Table 1); across all parasite exposures an average  
412 of  $27.3 \pm 15.14$  (SD) % of cercariae successfully encysted.

413

### 414 3.3. Growth and development

415 Larval mass of tadpoles at stage 38-40 (late stage) averaged  $917 \pm 140$  (SD) mg (N = 29)  
416 and the larval period to this stage averaged  $44.5 \pm 10.0$  (SD) d. Mass specific growth rate  
417 had a negative but non-significant correlation with number of metacercariae recovered  
418 postmortem ( $R^2 = 0.11$ ,  $p = 0.081$ , Fig. 1a.). Furthermore, larval mass at stage 38–40 ( $R^2$   
419  $= 0.02$ ,  $p = 0.392$ ) was not significantly correlated with the metacercariae intensity. The  
420 duration of the larval period to this stage was positively correlated to the number of  
421 metacercariae ( $R^2 = 0.33$ ,  $p = 0.001$ , Fig. 1b.), with each metacercaria adding  $\sim 0.25$  day  
422 to development.

423 Tadpoles weighed immediately prior to metamorphosis (stage 42) averaged  $979 \pm$   
424  $172$  (SD) mg (N = 43) and the duration of the larval period to this stage averaged  $53.7 \pm$   
425  $5.7$  (SD) d. Mass-specific growth rate ( $R^2 = 0.00$ ,  $p = 0.849$ , Fig. 1a.) and final larval  
426 mass ( $R^2 = 0.00$ ,  $p = 0.894$ ) were not significantly correlated with the number of  
427 metacercariae. Similarly, there was no significant relationship between developmental  
428 period to stage 42 and number of metacercariae ( $R^2 = 0.06$ ,  $p = 0.127$ , Fig. 1b.).

429

### 430 3.4. Metamorphosis

431 The duration of metamorphic climax varied widely (Table 2) and was positively  
432 correlated to tadpole mass at the initiation of climax ( $p < 0.001$ ), but not to the number of  
433 metacercariae recovered postmortem ( $p = 0.611$ ). The final mass of tadpoles at stage 46

434 was positively correlated to mass at initiation of climax ( $p < 0.001$ ), marginally  
435 negatively correlated to the duration of climax ( $p = 0.057$ ), and not related to the number  
436 of metacercariae ( $p = 0.573$ ). Tadpoles lost approximately one-third of their total mass  
437 during metamorphosis (Table 2). Mass loss showed a positive correlation with tadpole  
438 mass at initiation of climax ( $p < 0.001$ ) and a marginally significant positive correlation  
439 with duration of climax ( $p = 0.058$ ), but no relationship to the number of metacercariae ( $p$   
440  $= 0.821$ ). The percentage of mass lost during metamorphosis was not related to either the  
441 number of metacercariae recovered postmortem ( $p = 0.620$ ), or initial mass ( $p = 0.391$ ),  
442 but was positively correlated to the duration of metamorphic climax ( $p = 0.033$ , Fig. 2a).  
443

#### 444 3.5. Energetics

445 Late-stage (Gosner 38–40) tadpoles used for estimation of SMR had an average mass of  
446  $918 \pm 150$  (SD) mg ( $N = 22$ ). The average SMR of all late stage tadpoles was  $0.088 \pm$   
447  $0.018$  (SD) mL $O_2$ /h. The number of metacercariae encysted did not significantly affect  
448 SMR ( $p = 0.437$ ), but SMR was positively related to tadpole mass ( $p = 0.008$ ). To obtain  
449 the constants for the calculation of maintenance energy for metamorphic climax, we also  
450 generated a second simplified regression model including only  $\ln$ -transformed mass and  
451 SMR (because encystment was non-significant in the full model) of the late-stage  
452 tadpoles that showed a significant positive correlation ( $R^2 = 0.30$ ,  $p = 0.009$ ). The  
453 equation approximating this relationship was:  $\ln(\text{SMR}) = -3.3571 + 1.4149 \ln(m)$ .

454 During metamorphic climax, tadpoles maintained an average metabolic rate of  
455  $0.130 \pm 0.024$  (SD) mL/h ( $N = 28$ ), which resulted in an average total of  $20.10 \pm 6.12$   
456 (SD) mL  $O_2$  consumed (Table 2). The metabolic rate was variable during climax, but no

457 trends corresponded to time or any particular developmental stages. Instead, cumulative  
458 oxygen consumption increased linearly. The number of metacercariae did not  
459 significantly affect total ml of O<sub>2</sub> consumed during metamorphosis ( $p = 0.278$ ). However,  
460 both initial mass ( $p < 0.0001$ ) and duration of climax ( $p < 0.0001$ ) were positively  
461 correlated with total ml O<sub>2</sub> consumed. *Lithobates sylvaticus* tadpoles required an average  
462 of 377.83 J of energy to complete the metamorphic transition, which was allocated into  
463 approximately 26% maintenance and 74% developmental energy (Table 2). The amount  
464 of energy allocated to development was positively correlated with tadpole mass at the  
465 initiation of metamorphosis ( $p < 0.0001$ ) and with the duration of climax ( $p < 0.0001$ ,  
466 Fig. 2b.), but not related to the number of metacercariae ( $p = 0.654$ ). The percentage of  
467 energy allocated to development was not correlated with the number of metacercariae ( $p$   
468 = 0.945), initial mass ( $p = 0.084$ ), or duration of climax ( $p = 0.189$ ).

469

#### 470 **4. Discussion**

471 Using a laboratory experiment to gradually expose tadpoles to a realistic range of  
472 infection intensities, we found that *E. trivolvis* metacercariae had a negative, but not  
473 statistically significant affect, on mass-specific growth rate. In addition, exposure led to a  
474 significantly longer period of development to stages 38-40. However, no significant  
475 effects of infection were observed during metamorphosis, supporting the idea that  
476 parasite effects are host-stage specific (Holland et al., 2007). While our results indicate  
477 that amphibian metamorphosis is a critical transition period with significant energetic  
478 costs, concurrent infection with *Echinostoma trivolvis* trematode metacercariae did not  
479 significantly alter these energetic costs. In comparison to other species, the energetic

480 costs of metamorphic climax in *Lithobates sylvaticus* correspond with costs reported for  
481 other ranid species, but may differ from *Anaxyrus terrestris*, which has a different life  
482 history strategy (Beck and Congdon, 2003). While most research addressing energetic  
483 trade-offs between parasite defense and host processes has focused on a narrow range of  
484 standardized conditions, our work contributes to the integration of physiology and  
485 ecoimmunology by considering parasite infection with simultaneous energetic demands  
486 of stage-specific developmental processes (Robar et al., 2011; Warne et al., 2011).

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

503           We predicted that parasite infection would increase energy use and delay  
504 development during metamorphosis, based on the significant contributions kidneys make  
505 to standard metabolic demands coupled with energetic costs of conditions like  
506 development (Steyermark et al., 2005; de Souza and Kuribara, 2006; Robar et al., 2011).  
507 In our study, metacercariae may not have increased host metabolic rates during  
508 metamorphosis if their presence does not interfere significantly with pronephros  
509 degradation (Fox, 1963). The maturing mesonephros may have compensated for any  
510 interference of kidney function or the biased distribution of metacercariae between  
511 kidneys may have reduced energy costs by localizing damage (Johnson et al., 2014).  
512 Furthermore, energetic costs of infection may only be apparent during metacercariae  
513 development (Lemly and Esch, 1984); however, in *L. palustris* tadpoles earlier in  
514 development, an energetic response was not detected during encystment of *E. trivolvis*  
515 (Orlofske et al., 2009). Building on this earlier study, we found that *E. trivolvis*  
516 metacercariae did not influence energetics of metamorphosis, the duration of  
517 metamorphic climax, final mass, change in mass, and percentage of initial mass lost  
518 during climax. If energetic costs of infection are related to immune function, the  
519 suppression of the immune response during amphibian metamorphosis related to loss and  
520 reorganization of tissues, as well as destruction of lymphocytes, could help explain the  
521 lack of observed energetic costs (Rollins-Smith 1998). Parasites, such as the trematodes  
522 *Clinostomum* sp. and *Ribeiroia ondatrae*, with different body sizes and pathological  
523 impacts, might be expected to have more significant energetic or developmental costs  
524 prior to and throughout metamorphosis and would be useful models for future



525 investigations of energetic costs of parasitism in larval amphibian hosts (Blaustein et al.  
526 2012; Koprivnikar et al., 2012).

527         By examining consequences of infection at two stages of host development, our  
528 research also assessed how pathology and parasite infection changes over time. After the  
529 initial exposure to cercariae, 28% and 19% of the tadpoles exhibited edema in the late  
530 developmental stage and metamorphosis experiments, respectively. Mortality was low  
531 and occurred during the infection procedure early in development and metamorphic  
532 climax. Both melanized cysts, occasionally surrounded by a fibrous capsule of host-  
533 derived tissue, and viable cysts were recovered from both late developmental stage  
534 tadpoles and metamorphs (Martin and Conn, 1990). The number of metacercariae  
535 recovered from both late developmental stage tadpoles and metamorphs was positively  
536 related to the total cercariae exposure. The slightly lower average percent metacercariae  
537 recovered after metamorphosis could be attributed to a longer time available for host  
538 immune responses to degrade cysts or a loss of cysts during the degradation of the  
539 pronephros during metamorphosis (Fox, 1963; Belden, 2006). Unmelanized, and  
540 potentially viable metacercariae were observed in mesonephros and the location of the  
541 degraded pronephros in metamorphs, supporting the conclusion that some cysts can  
542 survive the degradation of pronephros during metamorphosis (Fried et al., 1997;  
543 Theimann and Wassersug, 2000; Schotthoefer et al., 2003). This is in contrast to earlier  
544 studies where cysts were not recovered in the region of the pronephros post-  
545 metamorphosis (Belden, 2006).

546         Importantly, quantification of the energetic costs of amphibian metamorphosis  
547 contributes to our ability to compare costs across species and amphibian life history

548 strategies. The total energetic costs of metamorphosis in *L. sylvaticus* were 377.8 J at  
549 18°C in comparison to 424.5 J for *L. palustris* at 25°C (Orlofske and Hopkins, 2009), 904  
550 J for *Hoplobatrachus tigrinus* at 27°C (Pandian and Marian, 1985), and 50.3 J for  
551 *Anaxyrus terrestris* at 25°C (Beck and Congdon, 2003). Duration of climax and tadpole  
552 mass differed greatly among studies; however, qualitative comparisons using isometric  
553 relationships with mass and time (i.e., total energy use converted to J/g/hr) can be  
554 informative. This comparison yields very similar energy expenditure for *L. sylvaticus*  
555 (2.53 J/g/hr) in comparison to *L. palustris* (2.57 J/g/hr) and *H. tigrinus* (2.63 J/g/hr), the  
556 other members of the family Ranidae, which all differ from the toad *A. terrestris* (6.76  
557 J/g/hr). The amount of energy allocated to development for *L. sylvaticus* was  
558 approximately 74 percent, which is higher than both *L. palustris* and *A. terrestris*, which  
559 required 50 percent and 40 percent respectively (Beck and Congdon, 2003; Orlofske and  
560 Hopkins, 2009). Although total energetic costs are higher, large tadpoles complete  
561 metamorphosis more efficiently by using proportionally less total energy for climax than  
562 small tadpoles. However, in contrast to *L. palustris* and *A. terrestris* (Beck and Congdon,  
563 2003; Orlofske and Hopkins, 2009), the negative relationship between percent  
564 development costs and mass was not significant in *L. sylvaticus*, suggesting the efficiency  
565 associated with development at a larger body size was not as pronounced. The  
566 temperature used for measurements of *L. sylvaticus* may not have been the most efficient  
567 temperature for development and could also have contributed to the relatively long  
568 duration of climax, which was longer than the other Ranid species despite the smaller  
569 body size of *L. sylvaticus*. The duration of climax contributed significantly to the total  
570 energy and developmental energy expended, which further supports the conclusion that

571 more slowly developing tadpoles require more energy for metamorphosis (Orlofske and  
572 Hopkins, 2009).

573         Our study characterized developmental components associated with  
574 metamorphosis that may influence fitness. Interactions among duration of climax, initial  
575 mass, and final mass indicated that initial larval size significantly affects the length of  
576 metamorphic climax, change in mass, and the final metamorphic size. The duration of  
577 climax also influences final size, and the amount and percentage of mass lost. The size  
578 advantage large tadpoles maintained after completing metamorphosis may increase  
579 fitness through higher juvenile survival, reduced time to maturity, and increased  
580 fecundity (Semlitsch et al., 1988; Berven, 1988, 1990; Semlitsch and Gibbons, 1990;  
581 Scott, 1994; Beck and Congdon 1999; Beck and Congdon 2000; Boone and Bridges,  
582 2003; Orlofske et al., 2009; Todd et al., 2011, 2012). Therefore, developmental effects at  
583 early life history stages may have legacy effects for adult reproduction.

584         Overall, our research contributes to our knowledge of the physiological costs of  
585 parasitism concurrently with other demands, an important component of the  
586 ecoimmunology framework in disease ecology (Hawley and Altizer, 2010). While  
587 energetically costly, amphibian metamorphosis appeared to be unaffected by parasites  
588 acquired during aquatic larval stages. However, parasitism negatively affected time to  
589 developmental stages immediately prior to metamorphosis, suggesting that parasites may  
590 contribute to differential impacts depending on host age. Environmental influences must  
591 be accounted for when examining the effects of parasites on amphibian metamorphosis.  
592 For amphibians that breed in temporary or semi-permanent wetlands, metamorphosis  
593 often coincides with resource limitation and pond drying, conditions where the effects of

594 parasite infection may be more detrimental (Kiesecker and Skelly, 2001; Koprivnikar et  
595 al., 2014). Additional physiological and biochemical studies are needed to help clarify the  
596 mechanisms of how macroparasites, including *E. trivolvis*, affect their amphibian hosts  
597 and the potential interaction with environmental factors (Warne et al., 2011; Koprivnikar  
598 et al., 2012).

599

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609

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847

848 **Legends**

849

850 **Figure 1.** A. Regression of growth rate ( $\Delta \ln[\text{mg}] / \Delta \text{day}$ ) of individual laboratory-raised  
851 late stage (stage 38–40, Gosner 1960, N = 29, filled symbols) and metamorphs (stage 46,  
852 open symbols) *Lithobates sylvaticus* tadpoles on the number of *Echinostoma trivolvis*  
853 metacercariae recovered after three repeated exposures to cercariae 19, 29, and 39 days  
854 post-hatch. The relationship between growth rate and the number of metacercariae  
855 recovered was negative but non-significant for late stage tadpoles ( $R^2 = 0.11$ ,  $p = 0.081$ ),  
856 and non-significant for metamorphs ( $R^2 = 0.00$ ,  $p = 0.849$ ). B. Regression of duration of  
857 larval developmental period (days) of *L. sylvaticus* tadpoles from the first exposure of *E.*  
858 *trivolvis* cercariae to developmental stage 38–40 (N = 29, filled symbols) and 42 (N = 43,  
859 open symbols) on the number of metacercariae recovered from each tadpole. Regression  
860 line shows the significant relationship for the stage 38–40 tadpoles ( $R^2 = 0.33$ ,  $p = 0.001$ ).  
861

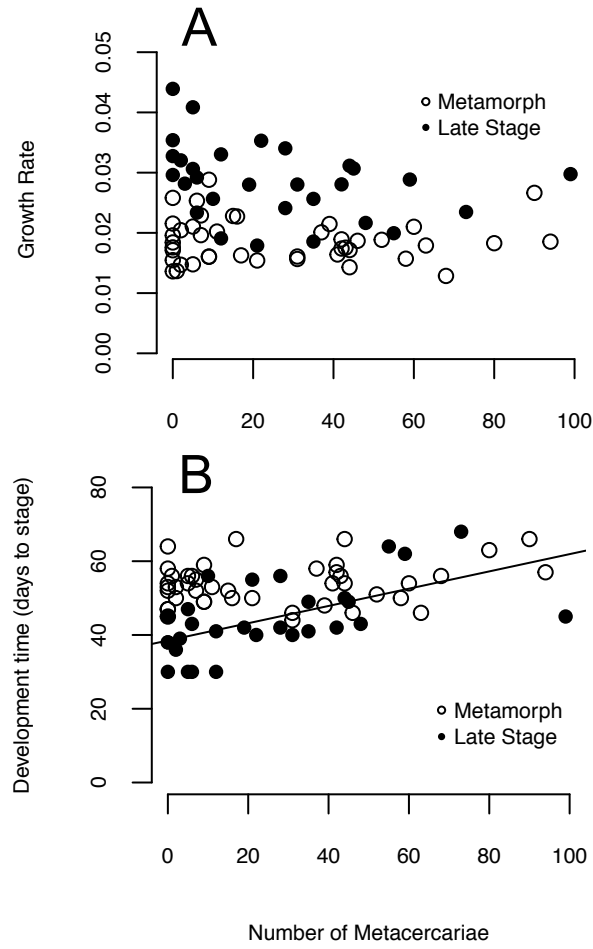
862 **Figure 2.** A. Regression of duration of climax (h) and the percent change in mass of  
863 tadpoles completing metamorphic climax ( $p < 0.0001$ , N = 43). B. Regression of the  
864 duration climax (h) and energy costs of development (J) ( $p = 0.033$ , N = 43).

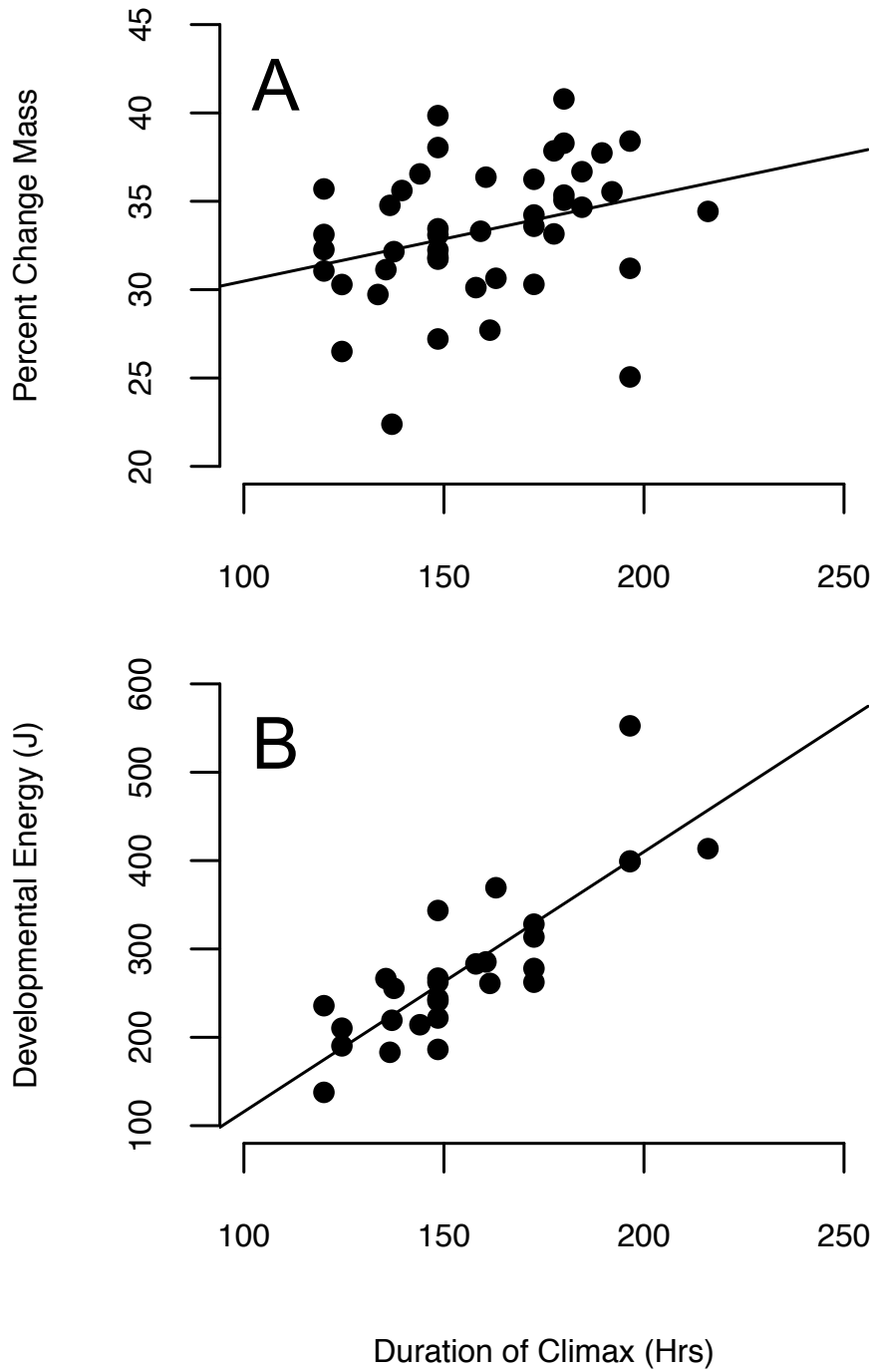
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867 Figure 1  
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872 Tables

873

874 **Table 1.** Percent encystment of *Echinostoma trivolvis* metacercariae in *Lithobates*  
875 *sylvaticus* tadpoles measured at late development (stage 38–40, Gosner 1960) and after  
876 metamorphic climax (stage 46) after gradual exposure to a range of cercariae (range of  
877 final exposure: 15–225) exposures in the laboratory occurring 19, 29, and 39 d post-  
878 hatch.

879

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Time	N*	Average %	SD	Minimum %	Maximum%
Late Stage	25	30.77	16.9	8.88	77.77
Metamorphs	37	24.34	15.0	0.00	60.00

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880 \*excluding controls

881

882 **Table 2.** Metamorphic climax data and associated energy requirements for individual  
 883 laboratory-raised *Lithobates sylvaticus* tadpoles. Because there was no effect of parasite  
 884 encystment on any response variables, data from different parasite exposure groups are  
 885 pooled here for descriptive purposes.  
 886

Variable	N	Mean	SD	Minimum	Maximum
Change in wet mass (g)	43	-0.327	0.071	-0.197	-0.462
Duration of climax (h)	43	159.2	25.2	120.0	216.0
% Change in wet mass	43	-33.31	3.95	-22.39	-40.79
Metabolic rate (mLO <sub>2</sub> /h) during climax	28	0.130	0.024	0.097	0.200
Total oxygen consumed (mL O <sub>2</sub> )	28	20.10	6.12	11.62	38.70
Total energy used (J)	28	377.83	115.10	218.55	727.50
Maintenance costs (J)	28	98.41	36.51	33.92	174.99
Developmental costs (J)	28	279.41	87.23	137.70	552.51
% of energy allocated to development (J)	28	73.99	6.28	63.01	88.28

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