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Abstract

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.

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

1. Introduction

Fundamental to understanding animal physiology is the concept of energetic trade-offs among the competing processes of growth, development, maintenance and reproduction (Stearns, 1989; Roff, 2001; Zera and Harshman, 2001; Lee, 2006). Amongst 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 (Warne et al., 2011, Blaustein et al., 2012). 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, temperature 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

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

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

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®, AquaScience Research
176 Group, Inc., North Kansas City, MO, USA) tap water (53.7 mg/L $CaCO_3$) and well water
177 (364 mg/L), to create a mixture with an acceptable hardness level of 108 mg/L of $CaCO_3$.
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 ± 1 SE of the tadpoles was 320 ± 9 mg
202 (N = 80), 504 ± 16 mg (N = 79), and 710 ± 21 mg (N = 78) at the first, second, and third
203 ceracariae exposures, respectively. The ranges of developmental stages (Gosner, 1960)
204 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 (stage 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 stage 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 stage 42 to 46), respiration, and loss of body mass during metamorphosis. Final mass was recorded for all individuals that reached stage 46 (N = 43).

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

al., 2004).

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 (stage 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 x 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 trails 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,

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_2 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_2 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_2
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_2 (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 $\ln(SMR) = a +$
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 (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 ± 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 ± 7.8 (SE) compared to 4.0 ± 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 ± 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 ± 140 (SD) mg (N = 29)
416 and the larval period to this stage averaged 44.5 ± 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 ~0.25 day
422 to development.
423 Tadpoles weighed immediately prior to metamorphosis (stage 42) averaged 979 ±
424 172 (SD) mg (N = 43) and the duration of the larval period to this stage averaged 53.7 ±
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

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 ($p = 0.391$), 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) mLO_2/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 ($R^2 = 0.30$, $p = 0.009$). 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 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_2 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_2 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

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 pathology 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 pervious study on *L. palustris* tadpoles (Orlofske et 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

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 ecoimmuniology 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).

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Legends

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

Figure 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 climax (h) and energy costs of development (J) ($p = 0.033$, N = 43).

867 Figure 1
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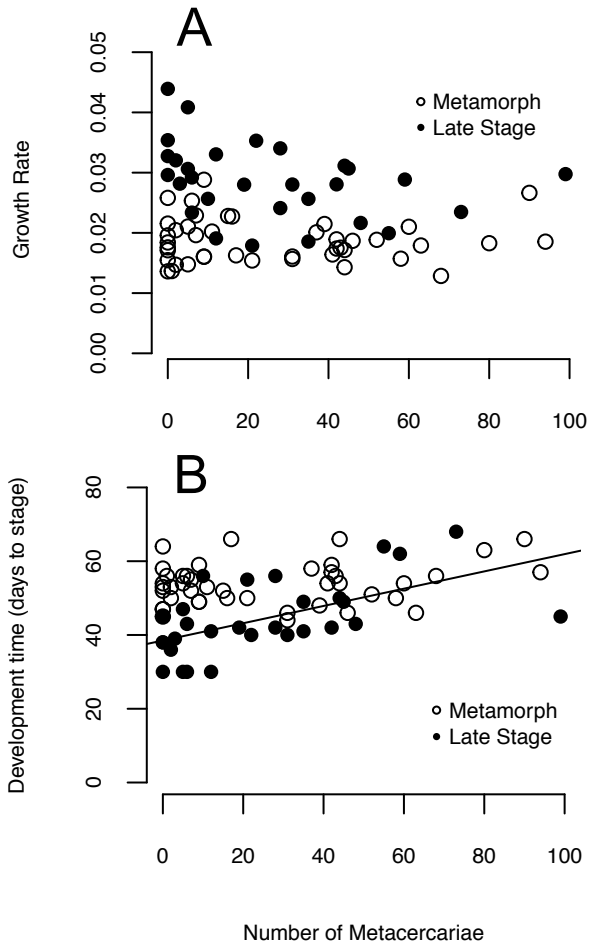
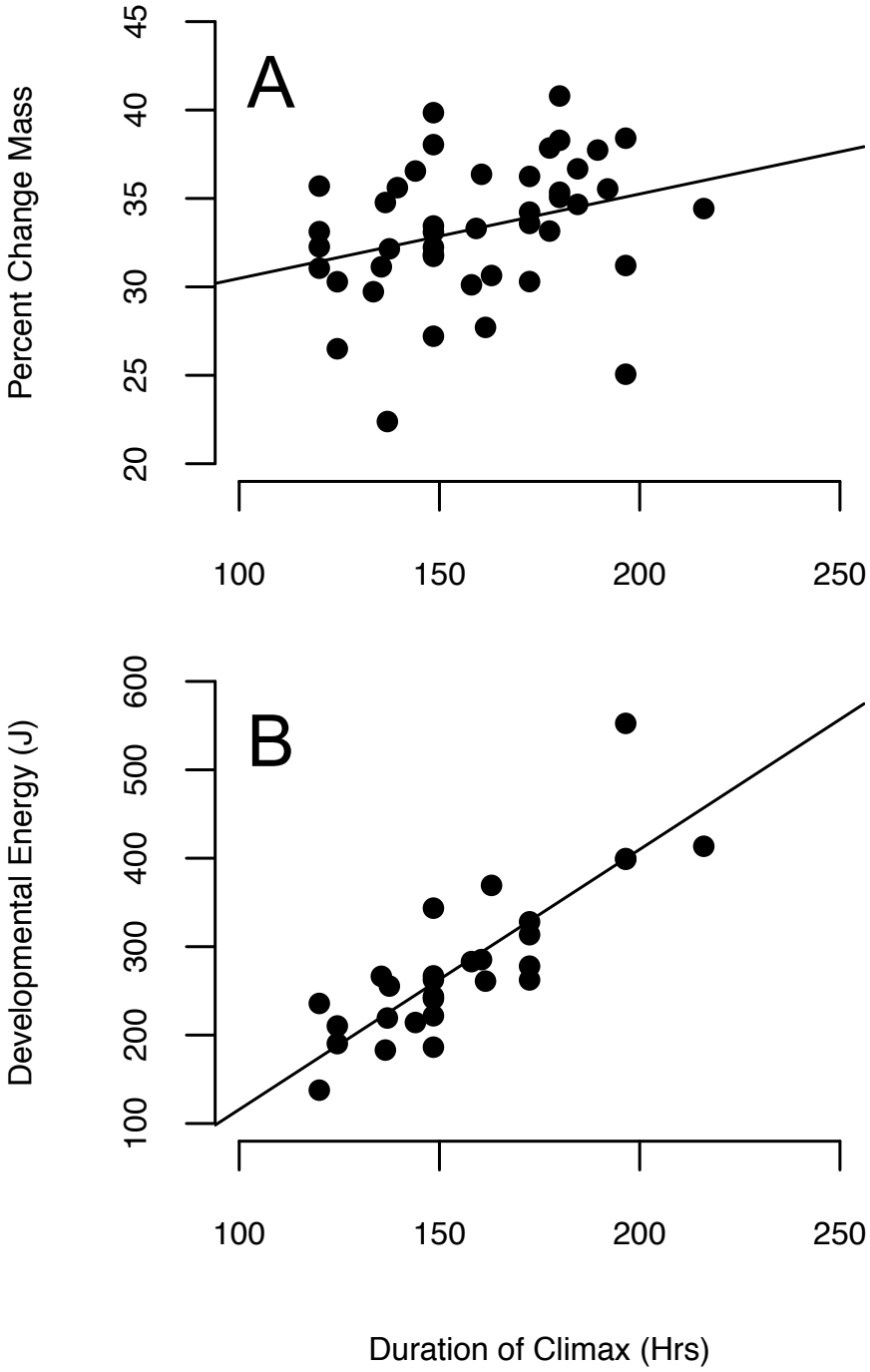


870 Figure 2



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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

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

880 *excluding controls
881

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

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 (mLO2/h) during climax	28	0.130	0.024	0.097	0.200
Total oxygen consumed (mL O2)	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