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RESEARCH ARTICLE

Exogenous stress hormones alter energetic and nutrient costs of development and metamorphosis

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ABSTRACT

Variation in environmental conditions during larval life stages can shape development during critical windows and have lasting effects on the adult organism. Changes in larval developmental rates in response to environmental conditions, for example, can trade off with growth to determine body size and condition at metamorphosis, which can affect adult survival and fecundity. However, it is unclear how use of energy and nutrients shape trade-offs across life-stage transitions because no studies have quantified these costs of larval development and metamorphosis. We used an experimental approach to manipulate physiological stress in larval amphibians, along with respirometry and ^{13}C-breath testing to quantify the energetic and nutritional costs of development and metamorphosis. Central to larval developmental responses to environmental conditions is the hypothalamic–pituitary–adrenal/interrenal (HPA/I) axis, which regulates development, as well as energy homeostasis and stress responses across many taxa. Given these pleiotropic effects of HPA/I activity, manipulation of the HPA/I axis may provide insight into costs of metamorphosis. We measured the energetic and nutritional costs across the entire larval period and metamorphosis in a larval amphibian exposed to exogenous glucocorticoid (GC) hormones – the primary hormone secreted by the HPA/I axis. We measured metabolic rates and dry mass across larval ontogeny, and quantified lipid stores and nutrient oxidation via ^{13}C-breath testing during metamorphosis, under control and GC-exposed conditions. Changes in dry mass match metamorphic states previously reported in the literature, but dynamics of metabolism were influenced by the transition from aquatic to terrestrial respiration. GC-treated larvae had lower dry mass, decreased fat stores and higher oxygen consumption during stages where controls were conserving energy. GC-treated larvae also oxidized greater amounts of ^{13}C-labelled protein stores. These results provide evidence for a proximate cause of the physiological trade-off between larval growth and development, and provide insight into the energetic and nutrient costs that shape fitness trade-offs across life stages.

KEY WORDS: Amphibian, Life history, Trade-off, Glucocorticoids, Corticosterone, Stable isotopes

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INTRODUCTION

Larval development is shaped by genetic and environmental interactions, and variation in conditions during critical developmental periods can have profound and lasting effects across an organism's life (Mueller et al., 2015). For example, larval developmental rates determine the period available for growth and, ultimately, body size and condition at metamorphosis – i.e. faster developing animals metamorphose at smaller sizes (Arendt, 1997; Nylin and Gotthard, 1998; Richter-Boix et al., 2011). This variation can have substantial consequences for fitness, because smaller metamorphic size is associated with decreased survival (Cabrera-Guzmán et al., 2013; Nylin and Gotthard, 1998; Tarvin et al., 2015) and lowered fecundity (Semlitsch et al., 1988; Taylor et al., 1998). Certain stages in larval life, including metamorphosis, are also critical windows of development, which are periods of heightened susceptibility for environmental factors to induce permanent phenotypic changes (Burggren and Warburton, 2005; Mueller et al., 2015). Directly quantifying costs of life-stage transitions could provide knowledge essential for understanding how environmental factors shape development and life history evolution (Crespi and Denver, 2005; Crespi et al., 2013; Stearns, 1992). Numerous studies have characterized metabolism during the few larval stages of metamorphic climax in aquatic free-swimming tadpoles (Beck and Congdon, 2003; Orlofske and Hopkins, 2009; Pandian and Marian, 1985; Wright et al., 2011), and one study quantified oxygen consumption across larval development of a direct developing amphibian (remained in the egg until metamorphosis; Mitchell and Seymour, 2000). However, no studies have taken an experimental approach to quantify both the energetic and nutritional costs of metamorphosis as well as the entirety of larval development for an aquatic, free-swimming species.

A physiological mechanism central to developmental processes, costs and responses to environmental stressors by amphibians and vertebrates is the hypothalamic–pituitary–adrenal/interrenal (HPA/I) axis. This axis is conserved across vertebrate taxa and the glucocorticoid (GC) hormones it secretes have been associated with developmental transitions across many taxa (reviewed by Crespi et al., 2013). The roles of the HPA/I axis are well described in amphibians (Denver, 2009). In late-stage larvae, GC hormones accelerate larval development, allowing them to escape suboptimal habitat by metamorphosing quickly (Crespi and Denver, 2005; Crespi and Warne, 2013; Denver, 2009). Such accelerated development results in smaller metamorphs that experience not only reduced survival, but also decreased adult fecundity unless they consume enough energy to undergo 'catch-up growth' in their first terrestrial year (Altwegg and Reyer, 2003; Berven, 1990; Boone, 2005; Cabrera-Guzmán et al., 2013; Goater, 1994; Hu et al., 2008; Scott, 1994; Scott et al., 2007; Semlitsch et al., 1988; Tarvin et al., 2015). This suggests a nutritional and/or energetic cost to accelerated metamorphosis that may be explained by pleiotropic effects of GC hormones.

In addition to developmental rate, GC hormones regulate metabolism and nutrient homeostasis. Activation of the HPA/I

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axis, and a subsequent increase in release of GC hormones, mobilizes glucose and lipid stores to meet increased energy demands (Sapolsky et al., 2000). Under conditions of chronically elevated GC levels, animals reduce tissue production and instead allocate resources to emergency homeostatic responses and somatic maintenance (Kirschman et al., 2016; Romero and Wikelski, 2010; Warne and Crespi, 2015). This may be particularly problematic for larval amphibians, as metamorphosis requires growth of new tissues and reorganization of existing ones, which can constrain biochemical pathways (Kirschman et al., 2016; Orlofske and Hopkins, 2009; Pandian and Marian, 1985; Shi, 2000). Restructuring of the digestive system in late-stage anuran larvae could exacerbate this energetic strain, as they undergo an obligatory fast and must complete the last stages of development with stored energy (Duellman and Trueb, 1994; Hourdry et al., 1996; Schreiber et al., 2005; Wright et al., 2011). This fast consumes a large portion of energy reserves, even in the absence of HPA/I activation (Beck and Congdon, 2003; Mitchell and Seymour, 2000; Orlofske and Hopkins, 2009; Pandian and Marian, 1985; Wright et al., 2011).

Survival of prolonged fasts depends on an animal's ability to store and allocate energy from diverse macronutrient stores (Lignot and LeMaho, 2012; McCue, 2010, 2012). While many studies of amphibian metamorphosis have indirectly quantified lipid stress to fuel metabolism, no studies have directly quantified varied lipid and protein substrate use in amphibians. An experimental approach combining carbon-13 (^{13}C) breath testing and GC hormone manipulation could greatly expand our understanding of how energetic costs and nutrient substrate use shift vary with physiological stress states in animals. Experimental approaches that manipulate GC levels in conjunction with direct measurements of energetic and nutrient use across development could provide insight into the proximate mechanisms that shape fitness trade-offs across life stages.

Amphibians are model organisms for exploring physiological mechanisms mediating larval conditions on development, life-stage transitions and fitness traits. Numerous studies have described metabolic patterns of metamorphosis and used indirect measures to estimate its energetic costs (Beck and Congdon, 2003; Sivula et al., 1972; Feder, 1982; Funkhouser and Foster, 1970; Funkhouser and Mills., 1969; Orlofske and Hopkins, 2009; Pandian and Marian, 1985). However, these studies have generally not taken an experimental approach to test how factors, such as physiological stress, may alter costs of life-stage transitions.

In this study, we measured energetic costs across larval development and metamorphosis in wood frogs [*Lithobates sylvaticus* (*Rana sylvatica*) (LeConte 1825)] exposed to exogenous GC hormones. We measured metabolic rates across larval ontogeny, and nutrient oxidation via ^{13}C-breath testing during metamorphosis, under control and stress-exposed conditions. To experimentally stress animals, we manipulated levels of the amphibian GC hormone corticosterone (CORT) in late-stage wood frog larvae. We predicted that exogenous CORT would increase developmental rates, reduce mass, increase metabolism, and deplete lipid and protein stores. To quantify nutrient usage for fuelling metamorphosis, we raised larvae on diets containing either a ^{13}C-labelled fatty acid (palmitic acid) or essential amino acid (leucine), whereby either their fat stores or protein-muscle stores were labelled, respectively. Through stable isotope breath testing, we could then measure larval lipid and protein oxidation during their obligate metamorphic fast. We predicted that CORT-treated larvae would oxidize more protein to complete metamorphosis, because they would have less energy stored as lipids.

MATERIALS AND METHODS

Animal collection and maintenance

We collected six wood frog eggs from a wetland complex surrounding Cave Creek in the Shawnee National Forest in Illinois, USA (37.641145 N, –89.340451 W), with permission from the United States Department of Agriculture Forest Service and under Illinois Department of Natural Resources permit number NH15.5778. The Institutional Animal Care and Use Committee at Southern Illinois University Carbondale approved all experimental procedures (15-007). Upon hatching, we haphazardly placed larvae from each clutch into three 50 l tanks. From these tanks, we randomly selected experimental larvae (Gosner stage 24; Gosner, 1960), which were housed in individual plastic containers with 500 ml of carbon-filtered water, fed rabbit pellets *ad libitum*, and kept between 24 and 27°C.

Energetics

We randomly sampled 10 larvae within every two Gosner stages (e.g. 24, 26, 28…) until completion of metamorphosis at stage 46 (n=120). At each stage, we measured oxygen consumption, excepting stage 24, as the larvae were too small and fragile. In aquatic stages (26–42), we measured oxygen consumption for 1 h at room temperature (26±1°C) via closed respirometry in centrifuge tubes with 58 ml of carbon-filtered water. We filled tubes entirely and capped them so there was no head space, and used a bubbler to saturate water with oxygen (>7.5 ppm) to prevent hypoxic stress. We measured dissolved oxygen at time 0 before introduction of an animal and at 60 min using a multi-parameter probe (Hach HQ40d, Loveland, CO, USA). We converted oxygen consumption into μmol h^{-1} for comparison between aquatic and terrestrial stages.

For oxygen consumption of terrestrial frogs (n=20), we used open-flow respirometry in an environmental chamber (Binder, Bohemia, NY, USA) kept at 26°C. Ten larvae were sampled at metamorphic climax (stage 44) and another 10 on the first day as juvenile frogs (stage 46). All animals remained unfed, because despite terrestrial morphology, larvae at metamorphic climax are still in an obligate fast and will not feed until completion of metamorphosis (Wright et al., 2011). Outside air was pulled through a dew point generator (DG-4, Sable Systems, North Las Vegas, NV, USA) to humidify the air to 80% relative humidity and then through 12 ml chambers at 30 ml min^{-1} by a subsampler (SS3, Sable Systems). We used a multiplexed system to analyse CO_2 (CA-10a, Sable Systems), intermittently sampling three animals for 20 min along with an empty baseline chamber for 10 min before and after each animal. Data were interpreted by universal interface (UI-2, Sable Systems) and recorded by Program Expedata (Sable Systems). Oxygen consumption rate ($\dot{V}_{O_2}$) was calculated with formulas from Lighton (2008). We converted oxygen consumption to μmol h^{-1} to facilitate comparison between aquatic and terrestrial stages. During these measurements, one of the chambers developed a leak, so we discarded all data from that chamber from our analyses; thus, total sample size was reduced from 20 to 15.

Following oxygen consumption measurements, we euthanized larvae and frogs in a 0.01% benzocaine solution and stored them at −80°C for later measurements of dry mass and lipid stores. We dried whole larval carcasses from stages 24/25 to 36/37 at 50°C before weighing. We dissected fat bodies from larvae of all stages 38/39–46 and dried them separately at 50°C to calculate lipid stores per unit body mass.

CORT treatment and metamorphosis

We focused only on Gosner stages that span the period of late prometamorphosis and metamorphic climax (38–46) to quantify

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energetic and nutrient costs of metamorphosis in relation to physiological stress. We exposed larvae at Gosner stage 36 to 62 nmol l^{-1} CORT in a 0.003% ethanol vehicle added to their aquarium water. We exposed 50 larvae and sampled 10 per stage for destructive sampling of metabolic measurements, body mass and body composition at each of the five Gosner stages (38, 40, 42, 44, 46). To induce a chronic elevation, we began CORT exposure at stages 36, and repeated the dosing every 48 h. A 62 nmol l^{-1} CORT dose induces an ecologically relevant elevation in whole-body CORT in amphibians (Glennemeier and Denver, 2002). We have found this dose also induces an ecologically relevant response in wood frogs (Kirschman et al., 2016) and significantly increases development rate (L.J.K., E. J. Crespi and R.W.W., unpublished). Eight measurements of terrestrial oxygen consumption for these CORT-treated larvae were removed from analyses because of the damaged chamber, leaving a sample size of 12.

^{13}C breath testing for nutrient store oxidization

Beyond metabolic measures, no studies have directly measured nutrient use during development and metamorphosis in amphibians. Most studies have relied upon destructive sampling to track changes in lipid composition; however, advances in stable isotope breath analysis allows for repeated measures on lipid and protein oxidation across ontogeny. Labelling macronutrient pools with ^{13}C allows measurements of rates of endogenous fuel oxidation (McCue, 2011). ^{13}C-labelled atoms are released as $^{13}CO_2$, which can be analyzed in exhaled breath, and can be used to investigate patterns of nutrient oxidation during fasting (McCue, 2012).

To quantify the nutrient costs of metamorphosis, we reared larvae on ^{13}C-labelled diets and sampled breaths at metamorphic climax (stages 43–45) when they transition to terrestrial respiration. We tracked nutrient oxidation by rearing larvae on one of two experimental diets labelled with ^{13}C. Using rabbit pellets as a base, we added either ^{13}C-labelled palmitic acid (1-13C, 99%; CLM-150-5) at 2.0 g ^{13}C-tracer per kilogram rabbit pellets or leucine (1-13C, 99%) at 1.0 g ^{13}C-tracer per kilogram rabbit pellets (Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA). The diets were tested by continuous-flow isotope ratio mass spectrometry at the Southern Illinois University Carbondale mass spectrometry laboratory to confirm enrichment of 20–40‰ above control (unlabelled) pellets. We reared larvae on these diets from hatching, thereby ensuring lipid or protein stores reached isotopic equilibrium with their respective diet (n=16 per diet). These macronutrient stores were presumed to have been primarily labelled by the respective ^{13}C-labelled nutrient owing to isotope routing. Routing is the process by which dietary nutrients (and their constituent isotopes) are routed into constrained biosynthesis pathways for tissue production of like nutrients (del Rio and Wolf, 2005). Palmitic acid, for instance, is the precursor of long chain fatty acid synthesis, and is not readily converted into carbohydrates or protein (McCue, 2011). Similarly, leucine and other essential amino acids are preferentially used in protein synthesis and not readily converted to carbohydrates or lipids (Kelly and del Rio, 2010; Newsome et al., 2014). Thus, we were able to quantify oxidization of either lipid stores derived from ^{13}C-palmitic acid, or protein stores derived from ^{13}C-leucine by measuring exhaled $^{13}CO_2$ from larvae (McCue and Welch, 2016).

We reared 16 larvae on each diet and split them into either a CORT or a control treatment as described above. Beginning at stage 43, when larvae are capable of terrestrial respiration (Crowder et al., 1998), we sampled exhaled CO_2 from each animal. To do so, we placed each animal into an individual airtight syringe with 15 ml of head space. At predetermined time points (4, 8, 28, 32, 52 and 56 h), we injected samples of respired air into evacuated vacuum-sealed vials (Exetainer, Labco Limited, Lampeter, UK). After sampling, each syringe was flushed with room air and resealed. We measured δ^{13}C values (international standard VPDB) of exhaled breath using a non-dispersive infrared spectrometer (HeliFan Plus, Fischer, ANalysen Instrumente, Germany) interfaced with an autosampler (FanAS, Fischer; methodology described in McCue et al., 2015). Breath δ^{13}C values were converted to rates of tracer oxidation using the methods of McCue et al. (2016). Briefly, we modelled δ^{13}C in atom fraction excess of larvae on enriched diets over those of larvae on the control diet and then calculated instantaneous rates of tracer oxidation in nmol h^{-1}.

We were unable to measure oxygen consumption simultaneously for breath-tested larvae, which is necessary to calculate tracer oxidation. Therefore, we estimated $\dot{V}_{O_2}$ using a regression model for our respirometry data. Because only two larvae reached stage 46 by the last day of breath testing, we restricted the regression to our data for stage 44 (R^2=0.75, P>0.0001, n=13).

Statistical analyses

To characterize rates of change in body mass and oxygen consumption ($\dot{V}_{O_2}$ as µmol h^{-1}) across larval development and metamorphosis, we fit separate joinpoint regression models (Joinpoint regression program, version 4.5, National Cancer Institute, Bethesda, MD, USA). We used Gosner stage as the independent variable in both models. We specified one minimum observation from an end joinpoint, one minimum observation between two joinpoints and zero points placed between adjacent observations. Both models required no minimum joinpoints, the highest maximum joinpoints allowed by the data (dry mass=5, $\dot{V}_{O_2}$=4), and used the Bayesian information criterion selection method (Martinez-Beneito et al., 2011).

We analyzed the effects of CORT treatment on larval dry mass and lipid content with a MANOVA (SAS University Edition, SAS Institute, Cary, NC, USA). Gosner stage and treatment (CORT versus control) were coded as nominal fixed variables in a full-factorial design. Dry mass was cube root transformed and lipid content was square root transformed to meet the assumption of homoscedasticity. We also analyzed the effects of CORT on metabolism with a general linear model (JMP 13 Pro, SAS Institute). Gosner stage and treatment were coded as nominal fixed variables in a full-factorial design and dry mass was added as a continuous covariate to account for body mass. We included stage 46 in the analysis because we were interested in the carryover effects of CORT on the first juvenile stage.

We analyzed leucine oxidation, palmitic acid oxidation and wet mass with separate general linear models. Because several measurements did not reach required levels of CO_2, we used average daily measurements (i.e. hours 1–4 and 4–8=day 1, hours 24–28 and 28–32=day 2, hours 48–52 and 52–56=day 3). In each model, treatment and day were coded in a full-factorial design with Gosner stage of the larvae as a covariate to account for differences in developmental rate. We used an autoregressive covariance structure with day coded as the repeated measure and larval identification numbers as subject designations. We compared developmental rates of breath-tested larvae using an ordinal logistic fit, comparing Gosner stage on the final day by treatment, because not all larvae reach stage 46. Finally, we analyzed larval survival curves between treatments with a log-rank test. We performed all analyses on breath-tested larvae in JMP 13 Pro (SAS Institute).

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RESULTS

Energetics

The joinpoint regression on dry mass identified a model with three joinpoints as the best fit (lowest Bayesian information criterion), thereby dividing the life cycle into four distinct metamorphic states (Fig. 1A). These fit the metamorphic states described by McDiarmid and Altig (1999). Stages 24–26: embryonic growth, which ends with closing of the operculum. Stages 26–34: premetamorphosis, characterized by growth of tail and trunk. Stages 36–40: prometamorphosis, dominated by growth and development of limbs and rising thyroxine titers. Stages 40–44: metamorphic climax, remodelling, and degeneration of larval tissue and onset of obligate fasting. The best-fit joinpoint regression model for oxygen consumption identified only two joinpoints (Fig. 1B). This is due, in part, to the lack of data for stage 24. However, a joinpoint was created by a reduction in oxygen consumption at stage 42 and a subsequent increase in terrestrial stages.

CORT treatment and metamorphosis

CORT treatment had a significant effect on larval dry mass ($F_{1,96}$=84.4, P<0.0001) and lipid stores ($F_{1,96}$=37.7, P<0.0001). Control larvae were heavier (Fig. 2A) and had more lipid stores relative to their mass (Fig. 2B). Gosner stage also had a significant effect on dry mass ($F_{4,96}$=33.0, P<0.0001) and lipid stores ($F_{4,96}$=10.0, P<0.0001). The treatment×Gosner stage interaction did not significantly affect dry mass ($F_{4,96}$=2.2, P=0.08) or lipid stores ($F_{4,96}$=1.2, P=0.31).

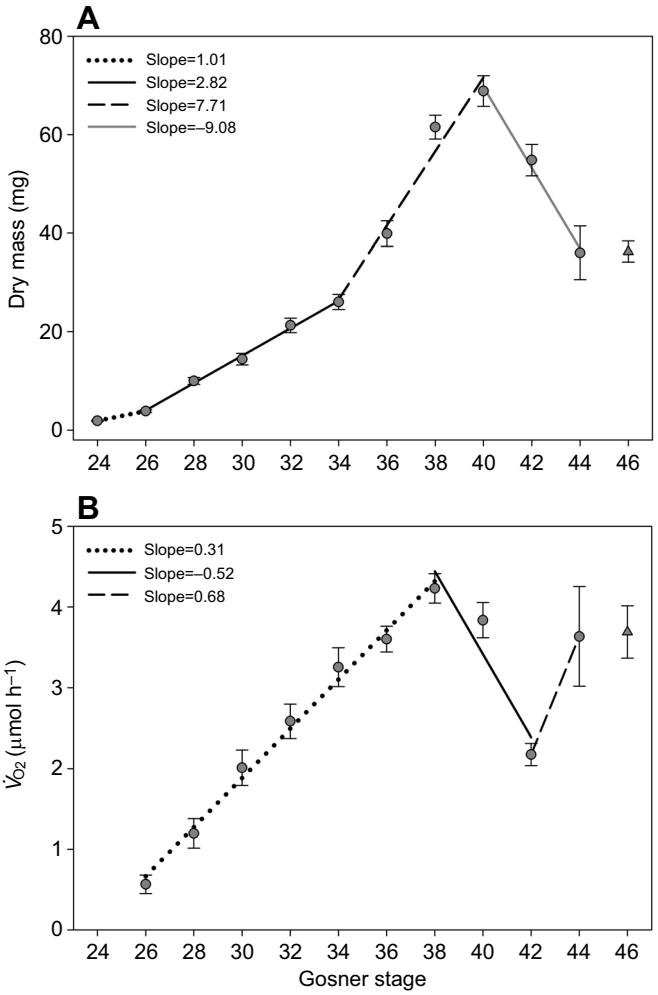

Fig. 1. Changes in mass and metabolism across the larval life-cycle. Joinpoint analysis of dry mass (A) and oxygen consumption (B) through the larval life cycle of control larvae (circles). Juvenile frogs (stage 46; triangles) are shown for comparison, but were not included in the analysis.

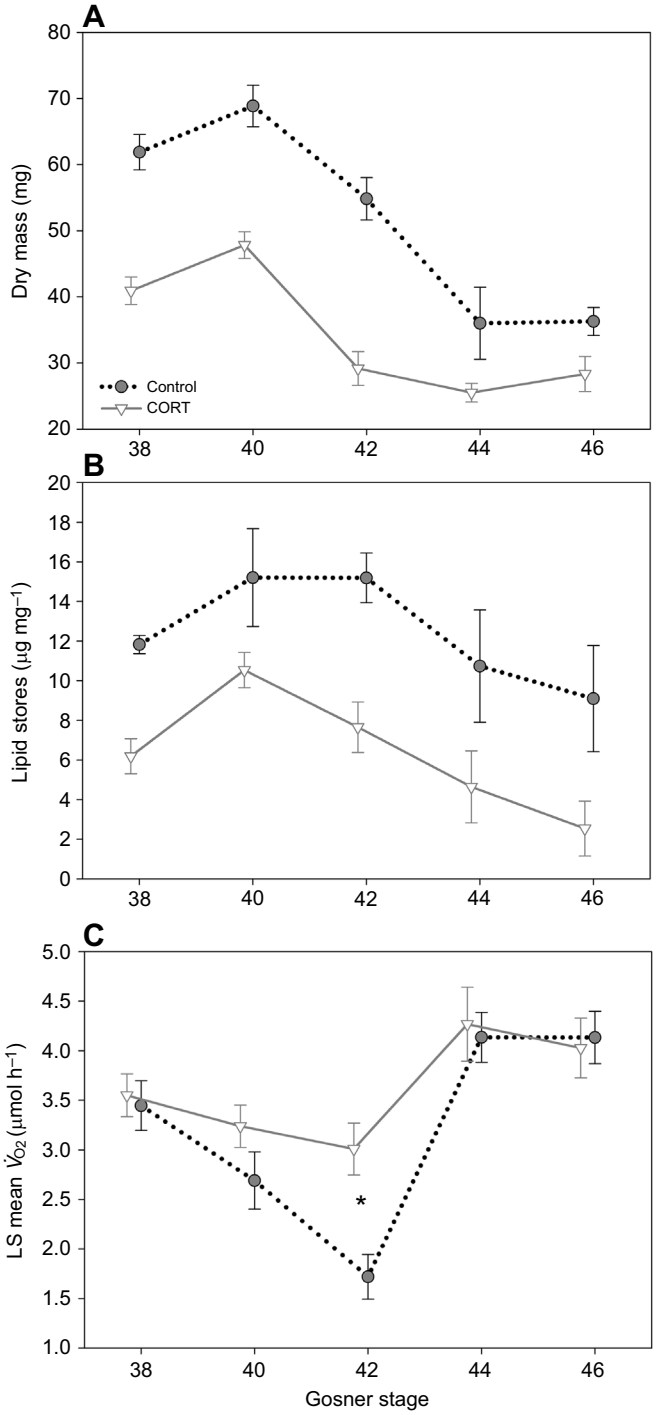

Fig. 2. Changes in larval mass, lipid reserves and metabolism during metamorphic climax. Larval dry mass (A), lipid stores (B) and the least squares means of oxygen consumption from a model with dry mass as a covariate (C) for control and corticosterone (CORT)-treated larvae. Control animals had significantly higher dry mass and lipid stores across all stages. CORT animals had significantly higher $\dot{V}_{O_2}$ at Gosner stage 42 (Tukey's HSD, *P<0.05). Points are offset for clarity.

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Larval oxygen consumption was significantly affected by the CORT treatment×Gosner stage interaction ($F_{4,83}$=2.7, P=0.04). CORT-treated larvae consumed more oxygen at stage 42 (Tukey's HSD, P<0.05; Fig. 2C). Gosner stage ($F_{4,83}$=17.4, P<0.0001) and dry mass ($F_{1,83}$=34.6, P<0.0001) also had significant effects. CORT treatment had no significant effect ($F_{4,83}$=0.1, P=0.76).

^{13}C breath testing for nutrient store oxidization

Protein oxidation was significantly affected by the CORT treatment×time interaction ($F_{1,19.6}$=5.0, P=0.04). CORT-treated larvae appeared to oxidize more protein on the first day (Fig. 3A). Treatment ($F_{1,12.0}$=0.9, P=0.35), time ($F_{1,20.6}$=0.6, P=0.45) and Gosner stage ($F_{3,15.2}$=2.2, P=0.13) did not significantly affect protein oxidation. Palmitic acid oxidation was not significantly affected by time ($F_{1,21}$=22.6, P=0.07), treatment ($F_{1,12.8}$=1.6, P=0.22), the treatment×time interaction ($F_{1,22.5}$=1.3, P=0.26; Fig. 3B) or Gosner stage ($F_{3,20.3}$=0.9, P=0.46).

Time had a significant effect on wet mass of breath-tested larvae ($F_{3,62.2}$=43.6, P<0.0001). In contrast, CORT treatment ($F_{1,38.1}$=3.3, P=0.08), the CORT-treatment×time interaction ($F_{3,69.1}$=0.5, P=0.50) and Gosner stage ($F_{3,60.8}$=1.8, P=0.16) did not have significant effects. Significantly lower survival of CORT-treated larvae (χ^2=4.7, d.f.=1, P=0.03) may have contributed to the lack of effect of CORT treatment on larval mass, as larvae in the worst body condition died. CORT-treated larvae developed faster, reaching higher Gosner stages by day three (χ^2=5.1, d.f.=1, P=0.02).

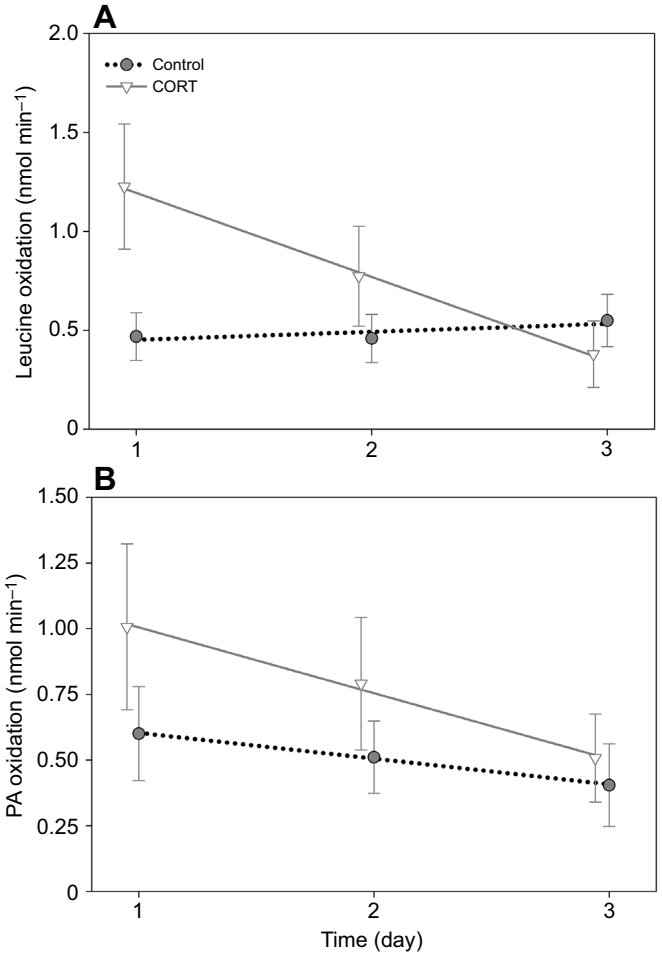

Fig. 3. Patterns of substrate oxidation during the obligate fast of metamorphic climax. Oxidation during metamorphic climax of lipid stores labelled with ^{13}C-leucine (A) and protein stores labelled with ^{13}C-palmitic acid (B). Symbols represent control and CORT-treated larvae. Larvae were sampled across 56 h during the developmental transition from Gosner stages 43 through 46 (juvenile frog). Leucine oxidation showed a significant treatment×time interaction (P=0.04). There were no significant effects on palmitic acid oxidation. Points are offset for clarity.

DISCUSSION

Our study provides the first report of how energetic costs and nutrient substrate use shift across larval development and metamorphosis under varying stress conditions in an amphibian. Generally, oxygen consumption correlated with mass and increased across development. At the beginning of metamorphic climax (stage 40), oxygen consumption dropped significantly, a pattern shown decades ago by Fletcher and Myant (1959). However, oxygen consumption fell much further than change in mass would predict at stage 42. This decline in oxygen consumption could be due to fasting-induced loss of specific dynamic action coupled with relative inactivity as well as shifts in mass and metabolic rates of restructured organs. During metamorphic climax, larvae restructure their digestive systems, enter an obligate fast (Hourdry et al., 1996; Schreiber et al., 2005) and are generally immobile unless disturbed. We observed a significant increase in oxygen consumption during the final phase of metamorphosis and transition to the terrestrial stages (Gosner stages 44–46), despite a decrease in mass. While some studies have quantified the total energy consumption across metamorphic climax (Beck and Congdon, 2003; Orlofske and Hopkins, 2009) and others have documented the rapid shift from aquatic to terrestrial respiration and the accompanying physiological changes (Burggren and Doyle, 1986; Crowder et al., 1998), ours is the first to record a substantial difference in oxygen consumption during this transition. This likely reflects the higher activity level of juvenile frogs (Pough and Kamel, 1984) fuelled by higher oxygen consumption relative to the environment. However, these dynamics were altered by exogenous CORT exposure at doses similar to an ecologically relevant stress response (Belden et al., 2005; Glennemeier and Denver, 2002; Kirschman et al., 2016; Rot-Nikcevic et al., 2005; Warne and Crespi, 2015), suggesting that exposure to environmental conditions that raise GC hormone levels can alter the energetic cost of metamorphosis.

Larvae exposed to exogenous CORT had lower mass and lipid stores, but consumed more oxygen during metamorphic climax relative to similarly sized controls. Throughout larval development, anurans stockpile lipid reserves to fuel metabolic processes during metamorphic climax and usually emerge as terrestrial frogs with some intact reserves (Gramapurohit et al., 1998; Wright et al., 2011). However, CORT-treated larvae had smaller lipid stores even before the start of metamorphosis (after 3 days of CORT exposure). Activation of the CORT response apparently shifted resources and energy allocation away from both growth and lipid storage. The energetic demands of metamorphic climax likely exhausted lipid stores of CORT-treated larvae. This conclusion is supported by the fact that 70% of CORT-treated larvae had no lipid reserves as juvenile frogs (stage 46), whereas all control larvae completed metamorphosis with lipid reserves. The CORT-treated larvae also maintained higher oxygen consumption rates at stage 42 relative to controls. This corresponded to a decrease in lipid stores before onset of the obligatory fast that was not evident in control larvae (Fig. 2B,C). Together, these data suggest that a CORT stress response acts to increase metabolism and lipid mobilization to cope with homeostatic challenges (e.g. pond drying, predation, disease) at a time when larvae would ordinarily conserve energy. CORT

levels are naturally elevated during metamorphosis, because this hormone binds to the maturing thyroid and interacts with thyroxine to regulate the timing of metamorphosis and responses to environmental conditions (Denver, 2009). However, our results suggest that sustained activation of the CORT response, especially during the critical developmental window of metamorphic climax, may have direct fitness consequences. Indeed, these results provide mechanistic evidence for the oft-cited fitness cost of increased developmental rates; for instance, lipid levels at metamorphosis are often linked to terrestrial survival and slower developing larvae have higher lipid reserves (Álvarez and Nicieza, 2002; Pfennig, 1992; Scott et al., 2007).

Beyond direct energetic costs of metamorphosis, our ^{13}C-breath testing results provide novel insights into shifts in nutrient use during metamorphic climax and in relation to a CORT response. During metamorphic climax (stages 43–46), oxidation of lipid stores labelled with ^{13}C-palmitic acid did not vary across the 56-h sampling period or between treatments (Fig. 3). However, given the smaller lipid stores of CORT-treated larvae, they may have exhausted lipid stores faster. This could explain why CORT-treated larvae oxidized greater amounts of protein stores labelled with ^{13}C-leucine early in metamorphosis, as they may need to shift to protein stores to fuel metabolism and complete the transition to the frog life stage. Sustained protein catabolism may present challenges for anuran larvae, because protein stores available for oxidation include muscle and newly formed adult structures crucial for terrestrial survival and developed at substantial energetic and nutritional costs (Beck and Congdon, 2003; Mitchell and Seymour, 2000; Orlofske and Hopkins, 2009; Pandian and Marian, 1985; Wright et al., 2011). In addition, during protein catabolism, the liver must deaminate amino acids, creating toxic ammonia, which larvae must excrete (Vikramjit and Metcalf, 2009). This may occur concurrently to liver remodelling necessary for the transition from ammonia to urea excretion (Brown and Cai, 2007). If these two processes together overburden the liver, toxic hepatitis may explain higher mortality in CORT-treated larvae and warrants further investigation. We did not measure nutrient metabolism of the earlier aquatic larval stages, but an increase in oxygen consumption coupled with a decrease in lipid reserves may indicate that CORT-induced accelerated development may also change substrate oxidation in earlier larval stages. The challenge with measuring nutrient oxidation in gill-breathing larvae precludes ^{13}C-breath testing, and would thus require alternative approaches in future experiments.

Cumulatively, these results provide evidence for a proximate cause of a physiological trade-off between larval growth and development. CORT-treated larvae were significantly smaller in the first measured stages following exposure. Like all animals, developing larvae cannot maximize all traits simultaneously and should preferentially allocate resources to traits that improve fitness (Perrin, 1992; Perrin and Silby, 1993; Stearns, 1989). However, activation of the HPA/I axis and release of GC hormones can alter and intensify these trade-offs (Boonstra et al., 2007; Crespi et al., 2013; Danstzter et al., 1992; Denver, 2009). In the case of amphibian larvae, particularly ephemeral breeders such as wood frogs, development rate and metamorphic speed are constrained by their niche; all larvae must escape annually drying ponds (Denver, 1997). Indeed, under food limitation, wood frog larvae allocate resources to maintain developmental rates at the expense of growth (Warne and Crespi, 2015). Viewed this way, small size at metamorphosis following HPA/I activation is not due to speed of larval development constraining time available for growth. Rather, HPA/I activation routes allocation towards development and away from growth, reducing time to and size at metamorphosis. This trade-off against growth may be further constrained because CORT increases metabolic rate, reducing energy stores. Furthermore, GC hormones have direct downstream effects on other systems (Crespi et al., 2013; Hawlena and Schmitz, 2010; Kirschman et al., 2016; Maher et al., 2013; Sapolsky et al., 2000); therefore, trade-offs between development and growth explicitly caused by increased GC hormones might be physiologically distinct from those caused by abiotic variables such as food limitation, temperature or intrinsic differences in growth rate. Given the effects of GC hormones on development across multiple taxa (Boonstra et al., 2007; Crespi et al., 2013; Danstzter et al., 1992; Denver and Crespi, 2006), future studies on development and growth should make efforts to differentiate cases facilitated by HPA/I activation and those due primarily to other abiotic factors.

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Competing interests
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