Corrigendum

# Stress hormones and the fitness consequences associated with the transition to a novel diet in larval amphibians

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10.1242/jeb.047639

There was an error published in *J. Exp. Biol.* **212**, 3743-3750.

In Fig. 4, the data for the top two graphs – depicting residual corticosterone regressed on residual mass – were plotted on inverted axes. The correct version of the figure is below.

The authors apologise for this error but assure readers that the results and conclusions of the paper remain unchanged.

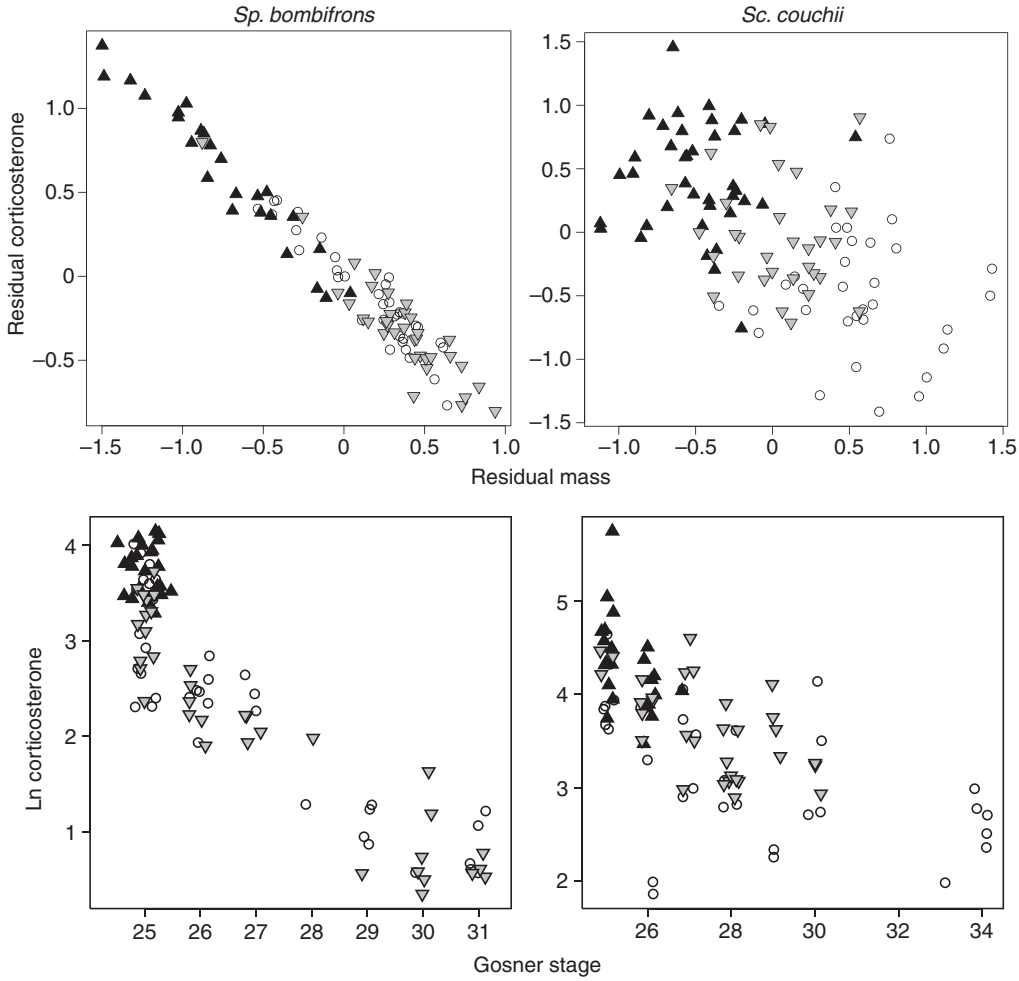


Fig. 4. The relationships of corticosterone to mass and developmental stage in *Sp. bombifrons* and *Sc. couchii* tadpoles. Data represent individuals from all time points and diets (open circles: detritus-fed; gray triangles: shrimp-fed; black triangles: unfed). For mass, the residuals of natural log-transformed mass and corticosterone (CORT) regressed separately on time (to remove the effect of time series) were regressed on each other. CORT decreased with increasing mass (*Sp. bombifrons*, $R^2_{adj}$=0.85, $P$<0.0001; *Sc. couchii*, $R^2_{adj}$=0.27, $P$<0.0001) and increasing stage (Helmert contrasts: *Sp. bombifrons*, $P$=0.05; *Sc. couchii*, $P$=0.002) in both species. Points along the developmental axes (bottom panels) were shifted left or right to improve visualization.

The Journal of Experimental Biology 212, 3743-3750
Published by The Company of Biologists 2009
doi:10.1242/jeb.034066

# Stress hormones and the fitness consequences associated with the transition to a novel diet in larval amphibians

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Accepted 10 August 2009

## SUMMARY

**Closely related species often specialize on different types of prey, but little is known about the fitness consequences of making an evolutionary transition to a novel diet. Spadefoot toad larvae provide a unique opportunity to reconstruct these evolutionary events. Although most anuran larvae feed on detritus or plankton, *Spea* larvae have also evolved the ability to consume large anostracan fairy shrimp. To investigate the changes that may have accompanied the shift to shrimp prey, we compared shrimp-induced physiological responses of *Spea* larvae with those of its sister genus, *Scaphiopus*, that has not made this transition. Although *Spea* larvae performed equally well on either diet, shrimp-fed *Scaphiopus* larvae experienced reduced growth and developmental rates, as well as elevated levels of the stress hormone corticosterone when compared with those that ate the ancestral detritus diet. These results suggest that ancestral *Spea* likely experienced reduced fitness when they first adopted a carnivorous feeding strategy.**

Key words: corticosterone, novelty, predation, anuran larvae, food restriction

## INTRODUCTION

Evolutionary diversification in animals is often accompanied by a change in resource use (Simpson, 1953; Schluter, 2000; Grant and Grant, 2008). For example, closely related, sympatric species typically differ in the prey on which they specialize, presumably as an evolutionary response that minimizes interspecific competition (reviewed by Schluter, 2000). Yet, such species must have had similar dietary preferences before they diverged, suggesting that shifts in resource use often accompany diversification. Despite the importance of dietary shifts, little is known about the physiological changes and fitness consequences that accompany them.

One way to assess the consequences of dietary transitions is to compare diet-induced responses in species or populations that exhibit an ancestral feeding strategy relative to those that have undergone a transition to a novel diet. Some studies that have taken this approach have found that populations may initially suffer a fitness cost as they make this transition. For example, Phillips and Shine (Phillips and Shine, 2006) found that when black snakes from Australia first encountered a novel prey item (introduced cane toads) the snakes probably suffered reduced fitness (cane toads are toxic). Black snakes were only able to prey consistently on cane toads once they had evolved behavioral and physiological traits that enabled them to cope with such prey. Furthermore, when organisms are presented with novel dietary resources, they may lack the behavioral, physiological or morphological characteristics for consuming that diet; thus a food-restricted condition either as a result of the inability to capture prey items or extract nutrients from prey items once ingested may be costly (e.g. Carroll et al., 1998). Whether such fitness costs are generally associated with dietary transitions is unclear.

We investigated the physiological changes associated with the transition to a novel diet in spadefoot toad larvae (Pelobatoidea). Most species within the Pelobatoidea clade feed on detritus, which is likely the ancestral diet of anuran larvae (Altig et al., 2007). According to character state reconstruction analysis, *Spea* is the only genus within the Pelobatoidea that has evolved the ability to exploit macroscopic prey (Ledón-Rettig et al., 2008). The evolution of carnivory in these tadpoles is associated with reduced intra- and inter-specific competition for food (Pfennig, 1992; Pfennig et al., 2007; Martin and Pfennig, 2009). Although this ecological scenario suggests potential fitness benefits to be gained from a carnivorous diet, especially when resources are limited, multiple characteristics are needed to sense, obtain, digest and assimilate nutrients from such prey. In order to better understand how ancestral populations of *Spea* may have shifted to a carnivorous diet, we examined the physiological changes that occur when larvae of *Scaphiopus couchii* Cope, a detritivore species in *Spea*'s sister genus (García-Paris et al., 2003), consume anostracan shrimp, a common prey item of modern *Spea* larvae (Pfennig, 1992; Pomeroy, 1981).

We hypothesize that the transition to a carnivorous diet in spadefoot tadpoles may have been associated with the inability of tadpoles to ingest or acquire nutrients from such a diet, thus causing nutrient stress that can have negative fitness consequences. Food restriction generates substantial morphological and developmental plasticity among anuran (Wilbur and Collins, 1973; Werner and Anholt, 1996; Kupferberg, 1997) and urodele (Wildy et al., 2001; Rohr et al., 2004) larvae. Generally, studies have shown that food restriction causes reduced body sizes at metamorphosis, but the effects on development time vary depending on when the restriction is experienced. During early developmental stages food restriction extends larval periods, as a minimum body size needs to be reached before metamorphosis can occur (Wilbur and Collins, 1973). By contrast, food restriction experienced during later developmental stages accelerates metamorphic timing [in spadefoots (Newman, 1987; Morey and Reznick, 2004)]. Spadefoot toads typically develop in short-lived ephemeral ponds that not only vary in initial resource

abundance, but also become depleted in resources as they dry (Pfennig, 1990; Pfennig et al., 1991). Given that growth and developmental rate are both under strong, positive, directional selection in spadefoots, reduced growth and development caused by nutrient restriction probably represents a fitness cost.

Physiologically, this plasticity in developmental timing and size at metamorphosis in response to food restriction (or other environmental stressors) is predominantly controlled by the neuroendocrine stress axis (reviewed by Denver, 2009). Recent studies in anuran tadpoles have shown that the stress axis is activated in response to food restriction (Crespi and Denver, 2005) or increased competition for food (Glennemeier and Denver, 2002a), resulting in increased corticosterone (CORT) content (the primary glucocorticoid in tadpoles), increased thyroid hormone secretion and accelerated development rates (Denver, 2009). Elevated CORT during food restriction has been observed in other vertebrates, and acts to inhibit growth while increasing gluconeogenesis and mobilizing energy stores from protein or lipid stores, to yield the necessary energetic resources needed to forage or seek more favorable conditions in the absence of nutrient inputs (Denver et al., 2002; McEwen and Wingfield, 2003).

In terms of diet transitions, the stress response to a novel diet could invoke morphological and physiological plasticity [i.e. cryptic phenotypic variation (Queitsch et al., 2002)] that facilitates the digestion of the novel food item. In spadefoot toads, for example, intestines of *Sc. couchii* tadpoles exposed to a novel shrimp diet shorten to look more like the guts of carnivorous tadpoles (Ledón-Rettig et al., 2008), thus sparing developmental resources that would otherwise be squandered on an 'unemployed' organ (Diamond, 1991). So while there may a short-term cost incurred by individuals ingesting a novel diet (e.g. reduced growth) the neuroendocrine stress axis could facilitate the evolution of a novel feeding strategy by exposing population variation in traits that would ultimately be adaptive for consuming that resource. Once exposed, this variation may become modified and refined by natural selection ['genetic accommodation', *sensu* West-Eberhard (West-Eberhard, 2003)].

To test the hypothesis that the neuroendocrine axis is activated when a carnivorous diet is introduced to larvae of spadefoot species with the ancestral feeding strategy, we exposed *Sc. couchii* tadpoles to shrimp and detritus diets from the time of hatching, and compared their response to that of *Spea bombifrons* Cope, a spadefoot toad species with a derived, carnivorous feeding strategy. We measured growth, developmental rate and whole-body CORT levels through ontogeny in these tadpoles to assess the physiological responses of each kind of tadpole to each diet. We predicted that *Sc. couchii* tadpoles would show reduced growth and development rates, and experience increased stress axis activity when exposed to shrimp. At the same time, we restricted the food of tadpoles of both species to assess whether growth, developmental and CORT responses to the novel diet were similar to complete nutrient restriction, or whether tadpoles were able to extract some nutrients and buffer the stress response. Finally, we experimentally manipulated glucocorticoid signaling in both species to determine the direct relationship between CORT and growth in these tadpoles. Ultimately, this set of experiments will resolve how the stress axis mediates the fitness costs incurred by these tadpoles when exposed to a novel diet.

## MATERIALS AND METHODS

All animals were collected near Portal, Arizona, USA. In this area, *Spea bombifrons* has undergone trophic character displacement with a closely related species, *Sp. multiplicata* Cope, making this *Sp. bombifrons* population particularly carnivorous (Pfennig and Murphy, 2000; Pfennig and Murphy, 2002; Pfennig et al., 2007). Despite the recurrence of shrimp from year to year in this area, *Scaphiopus couchii* feeds solely on detritus and microorganisms, although they will consume shrimp in the lab if it is the only available resource (Ledón-Rettig et al., 2008). Captured animals had been housed in a colony at the University of North Carolina, Chapel Hill for 1–2 years. To induce breeding, male and female adults were injected with 0.07 ml luteinizing hormone-releasing hormone (Sigma L-7134, St Louis, MO, USA) and left for 8 h in nursery tanks. All procedures were carried out in compliance with the Institutional Animal Care and Use Committee at the University of North Carolina, Chapel Hill, under application # 03-0110.

### Effects of diet on growth, development and whole-body CORT

One sibship each of *Sp. bombifrons* and *Sc. couchii* were bred for this experiment. After hatching (3 days after breeding), larvae from each sibship were transferred to individual 3 oz (~84 g) plastic cups, assigned a particular treatment, and then randomized and interspersed on racks in the same room maintained at 26°C and on a 14 h:10 h L:D photoperiod. In most anuran larvae feeding is precluded in early development by a plug of endoderm in the esophageal region (Wright, 2005). This plug is cleared in spadefoot larvae only after hatching, thus no food was administered during the first 3 days of development. On day 4 after breeding, larvae were fed either brine shrimp nauplii, ground fish food (hereafter, detritus), or received no food; brine shrimp resemble the fairy shrimp that *Spea* feed on in nature, whereas ground fish food resembles detritus in form and nutrition [fish food produces growth rates in *Spea* that are similar to growth rates found in natural ponds (Pfennig et al., 2006; Pfennig et al., 1991)]. Unfed tadpoles of both species were only raised until the third day of treatment. All other larvae were fed their respective diets, *ad libitum*, for the next 7 days. In the natural ponds where spadefoots breed and develop, anostracan shrimp undergo metamorphosis from nauplii to more complex, adult shrimp. Thus, 4 days after the initial feeding, both *Sc. couchii* and *Sp. bombifrons* tadpoles, which were initially fed nauplii, were switched to adult shrimp to emulate the natural development of shrimp in field conditions. An additional group of *Sc. couchii* tadpoles, initially fed nauplii, was switched to no food at all, to determine whether a shrimp diet presented any additional stress, beyond nutritional restriction, after 4 days. Yet another group of *Sc. couchii* tadpoles initially fed nauplii was kept on nauplii to control for the possibility that elevated CORT in *Sc. couchii* was due to the presence of the larger, more active adult shrimp, and not a shrimp diet *per se* (Fig. 1).

Tadpoles of both species were sampled 1, 2, 3 and 7 days after the onset of feeding. Each tadpole was transferred to a 12×75 mm Falcon tube, rapidly frozen by immersion in an ethanol and dry ice slurry, and transferred to a –80°C freezer. For each treatment (species:diet:time) 8–12 larvae were used. All samples were taken at the same time of day (approximately 21:00 h). Weight and developmental stage (Gosner, 1960) were recorded for tadpoles before they were individually transferred to glass tubes for radioimmunoassay (RIA).

Corticosterone was extracted from whole tadpoles following the method of Denver (Denver, 1998) with modifications. Briefly, total lipids were extracted by homogenizing each tadpole in 2 ml ethyl acetate; samples were centrifuged and the supernatant was reduced by rapid evaporation. Samples were spiked with 35,000 cpm tritiated CORT ([$^3$H]CORT, Perkin-Elmer, Waltham, MA, USA) to determine CORT migration distance on silica thin-layer

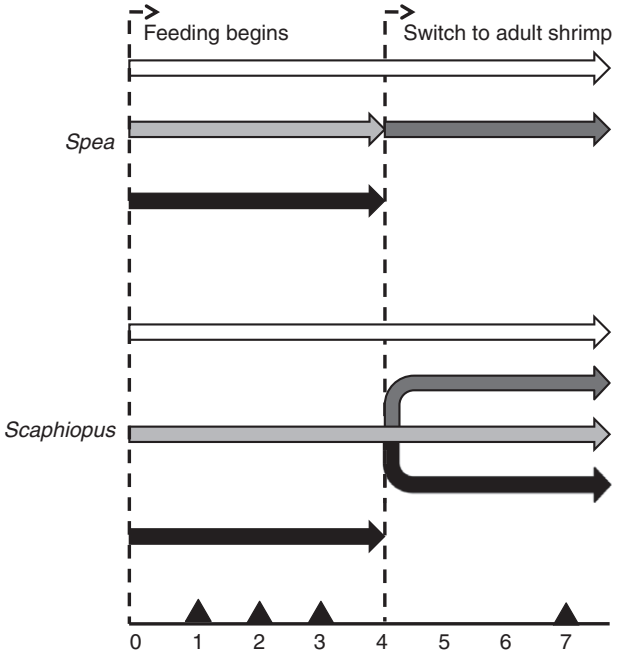


Fig. 1. Experimental design used to examine the effect of diet on *Sp. bombifrons* and *Sc. couchii* tadpoles in this study. One day after hatching both species were fed either detritus (white arrows), shrimp nauplii (light gray arrows), or nothing (black arrows). Tadpoles in the no-food treatment were only raised until 3 days after feeding. Four days after the initial feeding, both *Sc. couchii* and *Sp. bombifrons* tadpoles initially fed nauplii were switched to adult shrimp (dark gray arrows). Additional groups of *Sc. couchii* tadpoles initially fed nauplii were switched to no food at all or were kept on nauplii. Black triangles along the *x*-axis indicate days on which tadpoles were sampled.

chromatography (TLC) plates (JT Baker Si250F, Phillipsburg, NJ, USA) exposed to a toluene:cyclohexane (1:1) solvent system, followed by a chloroform:methanol (9:1) system (exposed twice). We located the CORT migration distance by scraping silica in 1 cm increments from two extra samples on the ends of the plate, and determining the radioactive peak using a Beckman LS6500 scintillation counter. We then scraped off the region of silica containing CORT from each sample, extracted CORT from the silica with 5 ml anhydrous ether, and dried samples under nitrogen (repeated to improve efficiency). Samples were resuspended in 0.5 ml 0.2 mol $l^{-1}$ phosphate-buffered saline with 1% gelatin for RIA. We included samples from each diet and time group on each TLC plate and RIA run; samples from each species were run on separate plates and assays. We conducted eight assays in total; the intra-assay coefficient of variation was 9.7%, and inter-assay variation was 11.5%.

### Statistical analyses

Because *Sp. bombifrons* and *Sc. couchii* vary drastically in size and developmental speed, species were considered separately for all described analyses. For each treatment (unique time point and diet), the sample size was eight for *Sc. couchii* and ranged from 8–12 for *Sp. bombifrons*. To determine the effects of diet on mass in each species, we performed ANOVAs with natural log-transformed mass as the dependent variable and assay, time, diet and the time by diet interaction as factors. We sequentially removed non-significant terms from each model, beginning with higher order terms. To identify specific pairwise differences, we used Tukey's test [*multcomp* package (Hothorn et al., 2008)] that included all combinations of time points and diets within each species (thus preserving the experiment-wise error rate at 0.05).

To determine how diet affected developmental rate in each species, we used non-parametric Pearson's $\chi^2$ tests since Gosner stage does not conform to parametric assumptions. Visual inspection of the developmental trends indicated that the most substantial differences in stage occurred 3 and 7 days after the onset of feeding (Fig. 2), so comparisons were made between diet groups collected at those times.

To determine the effect of diet on whole-body CORT, we performed ANCOVAs on each species with natural log-transformed CORT as the dependent variable. Assay, time, diet and the time by diet interaction as factors, and natural log-transformed mass and stage (as a categorical factor) were used as covariates. As with mass, we sequentially removed non-significant terms and performed Tukey's tests between all time points and diets to determine specific pairwise effects.

We also used linear regressions to determine the unique contribution of mass and developmental stage to whole-body CORT in our tadpoles. Species were analyzed separately, but data from all time points and diet treatments were considered. Because mass and CORT change over time, they can possibly generate a spurious correlation between each other (Yule, 1926). Therefore, to remove time-dependent trends, we regressed time with natural log-transformed mass and CORT, separately, and regressed the residuals of these models against each other. In a separate equation, to assess the contribution of development to whole-body CORT, natural log-transformed CORT was regressed on Gosner stages (transformed as Helmert contrasts, which allow for comparisons between qualitative groups that have an *a priori* ranking), and natural log-transformed mass (to control for the effect of mass).

All statistics were performed in the R 2.8.1 statistical language (R Development Core Team, 2008), except for the Pearson's $\chi^2$ tests, which were performed in JMP 7.0.1 (SAS Institute, Inc., Cary, NC, USA).

### Effect of corticosterone on growth

To determine whether variation in CORT causes changes in larval growth rate, we treated tadpoles of both species with exogenous CORT (Sigma, C2505) or a glucocorticoid receptor antagonist, RU-486 (mifepristone; Sigma, M8046). Solutions of 250 nmol $l^{-1}$ CORT and 100 nmol $l^{-1}$ RU-486 were prepared by first dissolving each solute in ethanol, and then diluting each in 0.1× Marc's modified Ringer solution (MMR; final dilution of ethanol: $0.25^{-3}$×). A 0.1× MMR and $0.25^{-3}$× ethanol solution was prepared for the control treatment. The concentrations of CORT and RU-486 were based on those used in previous studies (Crespi and Denver, 2004; Das and Brown, 2004), and were adjusted for the relative size and age of tadpoles used in our experiment. Because CORT and RU-486 are lipophilic compounds they are able to permeate tadpole tissues and cell membranes. Adding CORT to water in which tadpoles are housed has been shown to elevate their whole-body CORT with physiological effects (Krain and Denver, 2004).

One sibship each of *Sp. bombifrons* and *Sc. couchii* were used for this experiment. Two days after fertilization, 100 larvae were transferred to individual 3 oz plastic cups containing the previously prepared CORT, RU-486, or 0.1× MMR solutions. After 24 h, these individuals were fed 0.25 ml (approximately 10 mg dry mass) brine shrimp nauplii or 10 mg detritus. Cups were randomized and interspersed on racks at 26°C and on a 14 h:10 h L:D photoperiod.

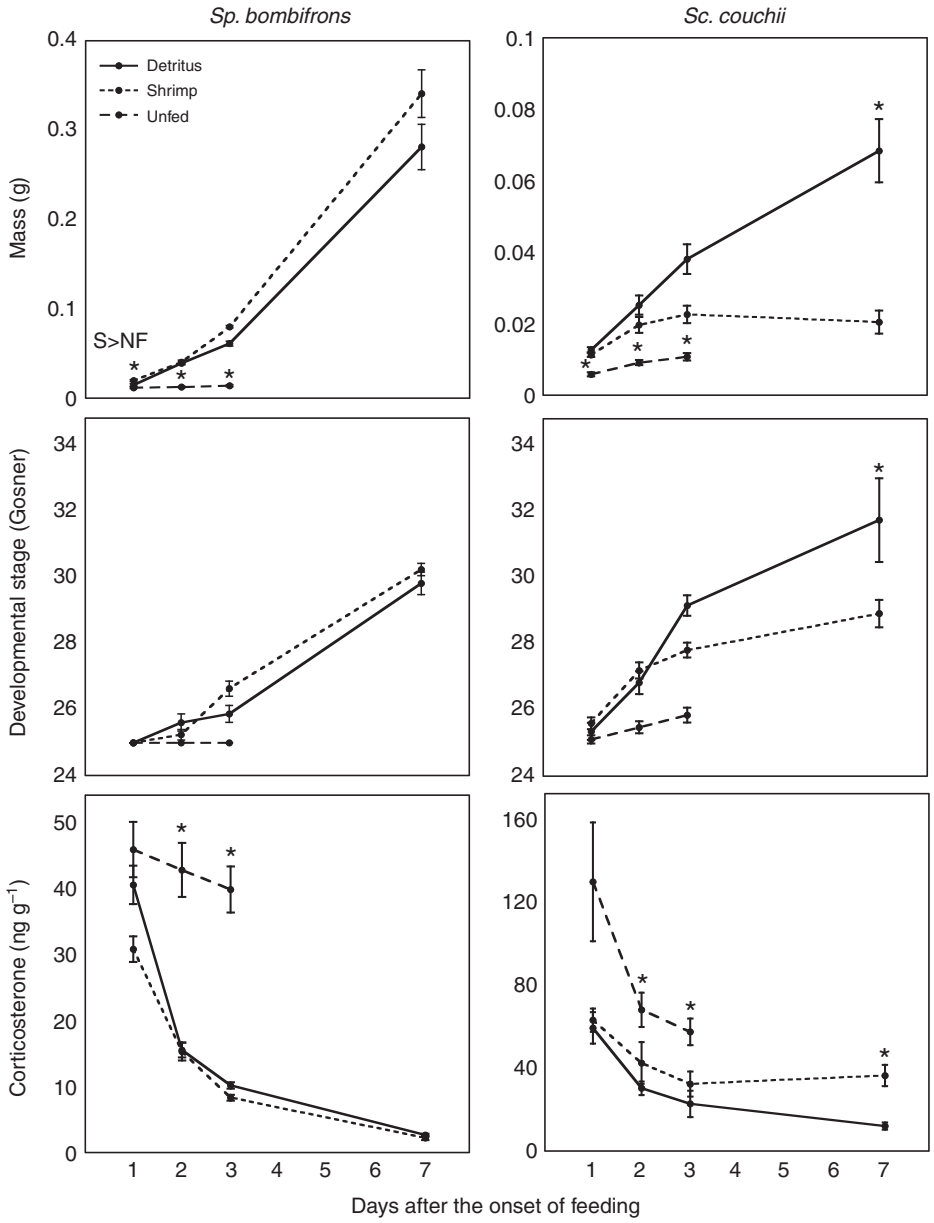


Fig. 2. Differential effects of diet on growth, development and corticosterone in *Sp. bombifrons and Sc. couchii* tadpoles. Asterisks denote significant differences between diets at the same time points ($P$<0.05, Tukey's HSD test for mass and corticosterone, Pearson's $\chi^2$ test for developmental stage), and bars indicate mean ± s.e.m. for each treatment group ($N$=8–12). Mean and s.e.m. for developmental stage are presented to facilitate visualization of the data, although non-parametric tests were used to statistically analyze this variable (see Materials and methods).

Twenty-four hours after feeding, tadpoles that had cleared their cups of food (ensuring that all analyzed individuals had consumed the same amount; $N$=12–24 per species:diet:hormone treatment) were euthanized with tricaine methosulfonate (MS-222), fixed in 4% paraformaldehyde, and rocked at 4°C overnight. Tadpoles were then dehydrated and stored in 100% ethanol at –20°C. A ventral photograph of each tadpole was captured with a Leica (Wetzlar, Germany) DFC480 R2 camera (magnification ×2.5), and the snout–vent length (SVL), a proxy for growth, was measured using NIH ImageJ (http://rsb.info.nih.gov/ij/).

### Statistical analyses

Separate ANOVAs were conducted for each species using natural log-transformed SVL as the response variable and hormonal treatment, diet and the hormone by diet interaction as the independent variables. The minimum adequate model was found by systematically eliminating non-significant terms, as previously described. Tukey's tests were then performed to determine whether hormonal treatments differentially influenced tadpole growth.

## RESULTS

### Effects of diet on growth, development and whole-body corticosterone

Mass in both species was influenced by assay, time, diet and a time by diet interaction (ANOVA: *Sp. bombifrons*, assay: $F_{(3,82)}$=8.45, time: $F_{(3,82)}$=440.24, diet: $F_{(2,82)}$=469.35, time by diet: $F_{(5,82)}$=15.98; *Sc. couchii*, assay: $F_{(3,87)}$=1.32, time: $F_{(3,87)}$=43.58, diet: $F_{(4,87)}$=63.25, time by diet: $F_{(5,87)}$=6.87, all $P$-values <0.0001). Unfed tadpoles grew poorly for the first 3 days of the experiment, weighing significantly less than fed tadpoles in both species (Fig. 2). Tadpoles fed either shrimp or detritus exhibited similar growth up to day 3 in both species, but thereafter, only *Sc. couchii* tadpoles diverged in a diet-dependent manner. *Sc. couchii* tadpoles fed detritus for 3 and 7 days grew more than similar tadpoles fed shrimp (Fig. 2). There were no differences in body mass between *Sc. couchii* tadpoles that were fed adult shrimp, nauplii, or no food between 4 and 7 days (Fig. 3).

Diet significantly delayed the development of shrimp-fed *Sc. couchii* tadpoles at 3 days (Pearson's test: $\chi^2$=7.62, $P$=0.05) and 7 days ($\chi^2$=24.00, $P$=0.0005) after the onset of feeding (Fig. 2). This

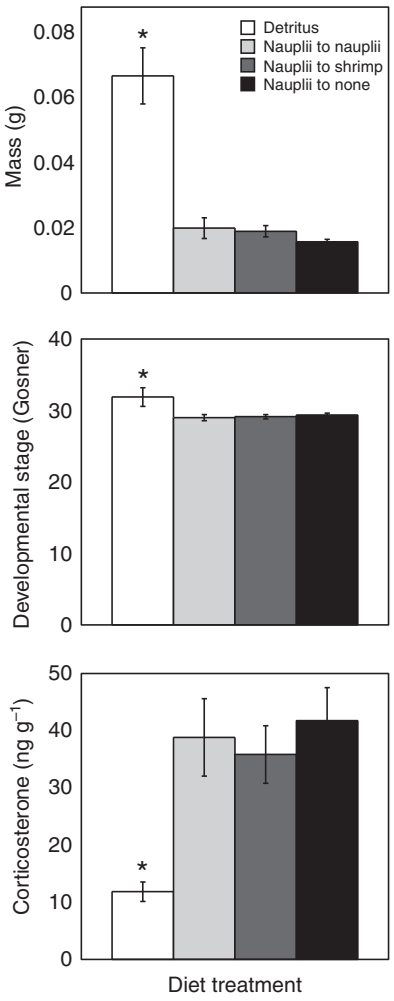


Fig. 3. Comparisons between the dietary shift to shrimp or food restriction on mass, development and whole-body corticosterone (CORT) in *Sc. couchii* tadpoles. Means ± s.e.m. for each variable are presented for tadpoles fed exclusively detritus or nauplii since hatching, those fed nauplii then adult shrimp at 4 days of feeding, and those fed nauplii then no food at 4 days of feeding ($N$=8/group). Asterisks denote significant differences ($P$<0.05, Tukey's HSD test for mass and CORT, Pearson's $\chi^2$ test for developmental stage). Mean ± s.e.m. for developmental stage are presented to facilitate visualization of the data, although non-parametric tests were used to statistically analyze this variable (see Materials and methods).

was not the case for *Sp. bombifrons* tadpoles, which showed no significant difference in developmental stage at either 3 days ($\chi^2$=4.67, $P$=0.20) or 7 days ($\chi^2$=6.48, $P$=0.09) from the beginning of feeding. Likewise, there were no differences in developmental stage between tadpoles that were feed adult shrimp, nauplii, or no food between 4 and 7 days (Fig. 3).

CORT levels in *Sp. bombifrons* were significantly influenced by mass, stage and assay (ANCOVA: $P$=0.02, $P$=0.03, mass: $F_{(1,80)}$=18,095.68, $P$<0.0001, stage: $F_{(8,70)}$=3.87, $P$=0.02, assay: $F_{(3,80)}$=100.75, $P$<0.0001) and marginally by diet and time (diet: $F_{(2,80)}$=2.62, $P$=0.083, time: $F_{(3,80)}$=2.31, $P$=0.08). Unfed *Sp. bombifrons* tadpoles generally had higher levels of CORT than fed tadpoles, but there was no difference between the CORT levels of shrimp- and detritus-fed tadpoles (Fig. 2). Although CORT was influenced by the same parameters in *Sc. couchii* (time: $F_{(3,78)}$=5.64, mass: $F_{(1,78)}$=264.35, $P$<0.0001, stage: $F_{(8,78)}$=3.87, $P$=0.0007, assay: $F_{(3,78)}$=31.02, $P$<0.0001, diet: $F_{(4,78)}$=7.46, $P$<0.0001), tadpole CORT varied in diet-dependent manner by 7 days after feeding began (time by diet interaction: $F_{(5,78)}$=4.02, $P$=0.003), with shrimp-fed tadpoles having significantly higher levels of CORT than detritus-fed tadpoles. We found no differences in CORT content between *Sc. couchii* tadpoles that were fed adult shrimp, nauplii, or no food from day 4 to 7 day (Fig. 3).

Mass had a significant effect on whole-body CORT in *Sp. bombifrons* ($F_{(1,95)}$=1,813.77, $R^2_{adj}$=0.95, $P$<0.0001) and *Sc. couchii* ($F_{(1,102)}$=28.62, $R^2_{adj}$=0.27, $P$<0.0001) tadpoles after removing the effect of time (Fig. 4). Likewise, developmental stage influenced CORT in both species (Helmert contrasts, *Sp. bombifrons*, $P$=0.05; *Sc. couchii*, $P$=0.002). When assessed individually, all contrasts between developmental stages in *Sp. bombifrons* were negative, and two were significant, indicating that CORT concentrations decline as these tadpoles develop. Similarly, in *Sc. couchii*, six of eight contrasts were negative, and three of these were significant (no positive contrasts were significant), again suggesting that CORT declines during early spadefoot development.

### Effect of corticosterone on growth

The results of the hormonal manipulation indicated that, regardless of food intake, glucocorticoid exposure affected growth in both species of tadpoles (Fig. 5; ANOVA: *Sp. bombifrons*, $F_{(2,110)}$=67.10; *Sc. couchii*, $F_{(2,105)}$=90.48, both $P$-values <0.0001). CORT significantly reduced growth in larvae of both species fed either diet after 2 days of exposure. By contrast, blocking glucocorticoid signaling with RU-486 significantly increased growth in *Sc. couchii* tadpoles. *Sp. bombifrons* tadpoles also tended to grow more when exposed to RU-486, but this effect was not significant ($P$=0.09).

## DISCUSSION

In order to investigate the changes that may have accompanied the evolutionary transition to a novel diet, we characterized growth, development and neuroendocrine stress response in two species of spadefoot toads – one that normally consumes only detritus and plankton (*Sc. couchii*) and a closely related species that can also consume shrimp (*Sp. bombifrons*) – when both species were given either an herbivorous or carnivorous diet. As predicted, *Sc. couchii* suffered growth and developmental costs when raised on shrimp but *Sp. bombifrons* did not. Also as predicted, and also in contrast to *Sp. bombifrons*, *Sc. couchii* experienced higher levels of whole-body CORT when raised on shrimp. These increased levels of CORT may be at least partially responsible for the reduced growth and development observed in shrimp-fed *Sc. couchii*.

Our findings suggest that the reduced growth and development rates of shrimp-fed *Sc. couchii* tadpoles were likely due to nutritional restriction. Tadpoles fed shrimp showed similar growth and development rates as those that were not fed. Because our tadpoles were fed *ad libitum*, we cannot say whether dietary restriction was caused by behavior (avoiding the diet) or the inability to assimilate nutrients once the diet was consumed. However, when forced to consume shrimp, most *Sc. couchii* tadpoles assimilate nutrients less efficiently than they will from the same amount of detritus (Ledón-Rettig et al., 2008). Therefore, at the very least, this species lacks the ability to digest or extract nutrients from shrimp at the level of the gut. Although it is not clear what traits aid *Spea* tadpoles in digesting shrimp (e.g. nutrient transporters or enzymes), they possess a number of behavioral and morphological features for consuming shrimp, thus rendering shrimp a higher quality diet. For example, compared with *Scaphiopus*, *Spea* tadpoles exhibit a stronger behavioral preference to eat shrimp (C.C.L.-R. and D.W.P., unpublished data), and develop enlarged jaw muscles and

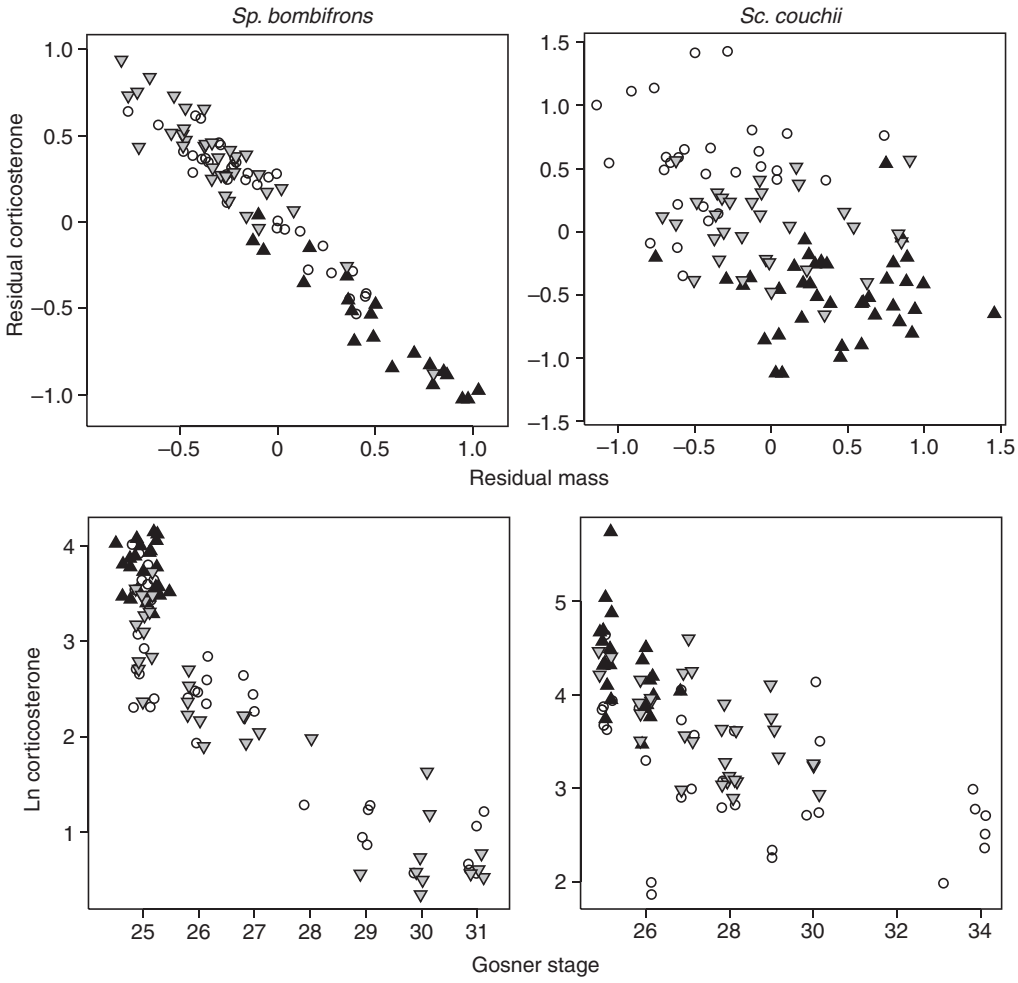


Fig. 4. The relationships of corticosterone to mass and developmental stage in *Sc. couchii* and *Sp. bombifrons* tadpoles. Data represent individuals from all time points and diets (open circles: detritus-fed; gray triangles: shrimp-fed; black triangles: unfed). For mass, the residuals of natural log-transformed mass and corticosterone (CORT) regressed separately on time (to remove the effect of time series) were regressed on each other. CORT decreased with increasing mass (*Sp. bombifrons*, $R^2_{adj}$=0.85, $P$<0.0001; *Sc. couchii*, $R^2_{adj}$=0.27, $P$<0.0001) and increasing stage (Helmert contrasts: *Sp. bombifrons*, $P$=0.05; *Sc. couchii*, $P$=0.002) in both species. Points along the developmental axes (bottom panels) were shifted left or right to improve visualization.

mouthparts for capturing shrimp (Martin and Pfennig, 2009). Presumably, these features make *Spea* tadpoles better able to capture and ingest shrimp, thereby maintaining growth and development.

In addition to reduced growth and attenuated development, the significant elevation of glucocorticoid production in shrimp-fed *Sc. couchii* tadpoles after 7 days of feeding suggests that these individuals were inefficiently extracting or assimilating nutrients from their diet. Although increased competition for food (i.e. increased density) and food deprivation elevates CORT levels in larvae of other anuran species [*Rana pipiens* (Glennemeier and Denver, 2002a); *Spea hammondii* (Crespi and Denver, 2005)], here we show for the first time that CORT level is affected by the quality of a given diet. In this case, the quality of diet is dependent upon a species' evolutionary history with that diet. This increase in corticosterone was likely stimulated by physiological factors indicating a state of negative energy balance and was associated with the mobilization of energy from stored fuels to keep these tadpoles functioning (McEwen and Wingfield, 2003). By 7 days of growth and development, the energetic demands of *Sc. couchii* tadpoles probably outstripped the nutrients they could extract from a shrimp diet such that development slowed, growth ceased, and CORT synthesis and secretion increased to a level similar to that of tadpoles that were not fed. The novelty of giving tadpoles adult shrimp after 3 days of eating nauplii did not appear to cause the stress response; tadpoles that were raised solely on nauplii also showed increases in CORT.

Although it appears that diet quality clearly affects mass and the activation of the neuroendocrine stress axis in *Sc. couchii*, it is not entirely clear which causal pathways link these variables. Our multiple regression analysis indicated that CORT concentrations varied inversely with body mass (and to a lesser extent with

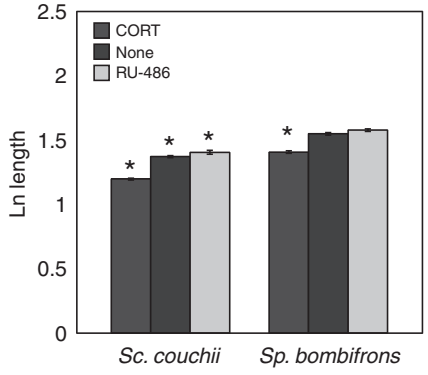


Fig. 5. The effect of glucocorticoids on growth of *Sp. bombifrons* and *Sc. couchii*. After hatching, tadpoles were transferred to 250 nmol $l^{-1}$ corticosterone (CORT), aged water or 100 nmol $l^{-1}$ RU-486. One day after hatching, tadpoles were fed either shrimp or detritus (diet treatments are pooled in the figure because there were no diet by hormone interactions). Asterisks denote significant differences (Tukey's test, $P$<0.05), and bars indicate ±s.e.m. for each treatment group ($N$=12–24).

development stage) in tadpoles, an outcome that is consistent with a recent study of wood frog tadpoles (Belden et al., 2007) and other vertebrates (Kitaysky et al., 1999; Moore et al., 2000). However, the effect of diet on *Sc. couchii* whole-body CORT levels after 7 days of feeding was significant even after statistically controlling for differences in tadpole mass. Thus, it appears that although diet quality has a distinct effect on body mass, additional physiological or central nervous system cues that signal nutrient restriction [e.g. neuropeptide Y (see Crespi et al., 2004)] or unique chemical cues from shrimp are probably stimulating the increases in CORT levels in tadpoles receiving a suboptimal diet. Furthermore, we showed experimentally that exogenous CORT decreases growth in both species, and in *Sc. couchii*, this decrease in growth was prevented by blocking CORT signaling with a glucocorticoid receptor antagonist. If CORT suppresses growth in *Sc. couchii* after 7 days of feeding (as it did in our exogenous hormone treatment), then the shrimp diet may have additional negative effects on body mass, either by stimulating an increase in metabolism or inhibition of growth factors associated with glucocorticoid signaling (McEwen and Wingfield, 2003).

Another interesting pattern that was revealed by our study was that the relationship between mass and whole-body CORT varied in the two species. *Sc. couchii* showed far more variation than *Sp. bombifrons* in this correlation, even within diet groups. It is unlikely that *Sc. couchii* larvae are more sensitive to environmental conditions, and that this variation is due to environmental differences among the individual cups in which they were raised. Previous studies have shown that species of *Spea* are similarly sensitive to even subtle environmental variation, such as water levels (Denver, 1998; Boorse and Denver, 2004; Gomez-Mestre and Buchholz, 2006). Furthermore, tadpoles of both species were collected at the same time and were spatially interspersed in this study, thus, if microenvironmental variation caused variation in CORT among *Sc. couchii* tadpoles, it should have done so among *Sp. bombifrons* tadpoles, as well.

An alternate possibility is that whole-body CORT is less coupled to mass in *Sc. couchii* as a result of their unique life history. For *Sc. couchii*, maintaining a constant developmental rate in spite of nutritional condition is essential for survival: the larvae of both species develop in ephemeral desert ponds, where the risk of mortality by desiccation is high (Bragg, 1965; Newman, 1987) but *Sc. couchii* tadpoles develop in ponds that are, in general, smaller and of shorter duration (Bragg, 1965). Possibly, as a result of this strong selection to metamorphose before their pond dries, *Sc. couchii* has evolved a developmental rate that is independent of food supply and, by extension, mass (Morey and Reznick, 2004). A tight link between whole-body CORT and mass might compromise the ability of *Sc. couchii* to metamorphose in time; thyroid hormone, which is responsible for metamorphic timing, and CORT are controlled by the same molecule [corticotropin releasing factor (Denver et al., 2002)]. Thus, relatively high variation in whole-body CORT content of *Sc. couchii* tadpoles may be the result of a relatively weak relationship between an individual's nutritional status and the neuroendocrine axis, which can vary between life stages in spadefoot toads (Crespi and Denver, 2005).

In addition to diet and mass, we also found that CORT content varied significantly with ontogeny. In both species, CORT content (per unit body mass) decreased dramatically with development during the early larval stages (stages 25–35). This pattern is similar to that of *Xenopus laevis*, where CORT is detectable just before hatching (stage 19), increases to its highest concentration (stage 26), and declines rapidly thereafter (Kloas et al., 1997; Glennemeier and Denver, 2002b). By contrast, in *Rana pipiens* or *R. catesbiana* CORT is low during early stages and becomes elevated as metamorphosis approaches (Krug et al., 1983; Glennemeier and Denver, 2002b). Spadefoot toads and *Xenopus* are more closely related to each other than either is to *Rana*, which may indicate that ontogenetic patterns of CORT are, to some degree, phylogenetically conserved. However, few studies have addressed the ecological and evolutionary causes of variation among amphibian neuroendocrine stress axes (Wada, 2008), and only additional studies encompassing a broad range of taxa will shed light on the origins of such variation.

### Conclusions and evolutionary implications

Because *Scaphiopus* is the most closely related taxonomic group to *Spea* that maintains the ancestral feeding strategy, it is in a unique position to shed light on the diet-induced responses of ancestral *Spea* populations. If ancestral *Spea* populations were like *Sc. couchii* in their growth, developmental and hormonal responses to shrimp, they may have had to surmount these physiological consequences as they transitioned to a novel diet. These physiological effects could, in turn, contribute to fitness costs, such as mortality resulting from insufficient food or small body size if tadpoles reach metamorphosis. A general issue requiring clarification is how taxa eventually offset these fitness costs. One evolutionary scenario is that populations may endure prolonged periods of reduced fitness before variation arises (through mutation, recombination, or introgression with another species) that facilitates the utilization of a novel resource. An alternative scenario is that natural selection may act on standing variation to produce a population that is specialized for the novel diet. Such standing variation may often be expressed (and, therefore, exposed to selection) through phenotypic plasticity, only after a population actually encounters a novel resource or environment (Waddington, 1959; Rutherford and Lindquist, 1998). Diet-induced stress hormones may be a mechanism of mediating the expression of this phenotypic variation. For example, it has been suggested that stress-induced thyroid hormone fueled developmental and morphological plasticity in ancestral groups of spadefoot toads – plasticity that was ultimately responsible for diversification among species (Gomez-Mestre and Buchholz, 2006). Future studies will determine whether *Scaphiopus* populations possess heritable variation in diet-induced CORT and its downstream effects on physiology, behavior and morphology. Demonstrating such heritable variation would suggest that diet-induced CORT played a substantial role in ancestral *Spea*'s transition to a novel diet.

We thank S. Glass for assistance with collecting and photographing tadpoles, J. Weiss for statistical advice, and E. Ketcham for assistance with laboratory work. We also thank R. Martin, D. Kikuchi and A. Leichty for their thoughtful comments on this manuscript. The valuable suggestions from two anonymous reviewers also improved an earlier version of this article. Funding for this study was provided by the National Science Foundation (IOS-0818212) to E.J.C. and (DEB-0640026) D.W.P, and a Graduate Research Fellowship to C.L.R. Funding for travel to Vassar College to conduct corticosterone analyses was supported by a Traveling Fellowship provided by *The Journal of Experimental Biology*.

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