

Roles of stress hormones in food intake regulation in anuran amphibians throughout the life cycle

Erica J. Crespi^{a,*}, Robert J. Denver^{a,b}

^aDepartment of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA

^bDepartment of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA

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Abstract

Towards understanding the ontogeny of energy balance regulation in vertebrates we analyzed the responses of corticotropin-releasing factor (CRF) and corticosterone to food deprivation in the Western spadefoot toad (*Spea hammondi*) at three developmental stages: premetamorphic tadpole, prometamorphic tadpole, and juvenile. Corticosterone responses to 5 days of food deprivation varied among developmental stages. Both pre- and prometamorphic tadpoles increased whole-body corticosterone content with food deprivation, but the magnitude of the response of premetamorphic tadpoles was significantly greater. By contrast, juvenile toads decreased plasma corticosterone concentration. Similarly, brain CRF peptide content tended to increase in food-deprived tadpoles but did not change in food-deprived juveniles. Therefore, there is an ontogenetic difference in the way the hypothalamic–pituitary–interrenal (HPI) axis responds to food deprivation in amphibians. In tadpoles, the HPI axis is activated in response to fasting as is seen in birds and mammals, and may be associated with mobilization of stored fuels and increased foraging. Juvenile toads do not respond to food deprivation by activating the HPI axis, but instead pursue a strategy of energy conservation that involves a reduction in plasma corticosterone concentration.

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1. Introduction

Corticotropin-releasing factor (CRF) and glucocorticoids are integral components of the hypothalamic–pituitary–adrenal (HPA) axis, or stress axis, and play important roles in the central regulation of food intake and energy balance (Heinrichs and Richard, 1999; Richard et al., 2002). Corticotropin-releasing factor has an inhibitory effect on food intake, as stress-induced anorexia is associated with an increase in CRF expression and the anorexia can be reversed by treatment with CRF receptor antagonists (Heinrichs and Richard, 1999). By contrast, short-term corticosterone treatment stimulates appetite in mammals, fish, and amphibians (Dallman et al., 1999; Tataranni et al., 1996; Bernier et al., 2004; Crespi and Denver, 2004a,b). The opposing actions of

CRF and glucocorticoids on food intake are evolutionarily conserved across vertebrates and are coordinated in an adaptive physiological response that maintains energetic homeostasis after an acute stressor (Sapolsky et al., 2000; Carr, 2002; Bernier et al., 2004; Crespi and Denver, 2004a,b). The central effects of hypothalamic CRF secretion associated with the immediate “fight or flight” stress response inhibit foraging and promote survival behaviors. After the threat passes, energy balance is restored through increased plasma glucocorticoid concentration, which stimulates appetite and reduces CRF expression via negative feedback (Sapolsky et al., 2000).

Almost all studies on the neuroendocrine controls of food intake have been conducted on juvenile or adult animals; thus, little is known about the roles of CRF and glucocorticoids in energy balance regulation in larval or fetal life stages. These controls are especially important in vertebrates with free-living larval forms, such as fish and amphibians.

* Corresponding author. Tel.: +1 734 647 2604; fax: +1 734 647 0884.

E-mail address: ejcrespi@umich.edu (E.J. Crespi).

Stress hormones also may be important in energy balance during fetal development of mammals, as suggested by the adverse effect of elevated maternal cortisol on fetal growth (Lesage et al., 2001; Jensen et al., 2002). Similarly, extended elevations of corticosterone during embryonic or larval stages also reduce growth in birds and amphibians (Hayes et al., 1993; Glennemeier and Denver, 2002c; Love and Williams, 2004; Hayward and Wingfield, 2004). Recent studies suggest that adult metabolic or energy balance disorders, such as type II diabetes and obesity, manifest from in utero effects of elevated glucocorticoids during fetal development (i.e., ‘fetal programming’; Breier et al., 2001; Brunson et al., 2001). These studies have resulted in heightened interest in understanding the ways by which stress hormones affect the development of neuroendocrine controls of energy balance and food intake.

Towards understanding the ontogeny of neuroendocrine regulation of energy balance and food intake in vertebrates, we studied the roles of CRF and glucocorticoids in appetite control in tadpoles and juvenile frogs (Crespi and Denver, 2004a,b). Most anuran amphibians exhibit complex life cycles with two completely different foraging strategies in the larval and postmetamorphic stages. Tadpoles are primarily filter-feeders or herbivorous grazers, but juvenile and adult amphibians are carnivorous predators (Larsen, 1992). Given this difference in foraging strategy, it is likely that the neuroendocrine controls of food intake differ between tadpoles and postmetamorphic amphibians. In addition to examining the roles of CRF and corticosterone in daily food intake regulation, we can directly manipulate the environment of the tadpole to determine how the neuroendocrine regulation of foraging behavior is altered by environmental stress. Such studies can yield essential insight into the development of neuroendocrine controls of feeding behavior and energy expenditure. Furthermore, the investigation of the neuroendocrine control of food intake in phylogenetically and ecologically diverse groups is necessary to understand the evolution of energy balance signaling pathways among vertebrates.

In the current paper, we present results from experiments in which we examined the response of brain CRF and plasma or whole-body corticosterone to food deprivation across three developmental stages of Western spadefoot toads (*Spea hammondi*). Based on ecological differences in life history stage, foraging mode, and growth rate, we predicted that tadpoles would mount a stress response to a decrease in resource availability, while juveniles would not. We place our findings in the context of our previous studies which examined the development of the hypothalamic–pituitary–interrenal (HPI) axis (see review; Denver et al., 2002; Glennemeier and Denver, 2002a,b,c; Boorse and Denver, 2003) and the ontogeny of neuroendocrine food intake controls in amphibians (Crespi and Denver, 2004a,b). We also compare the development of neuroendocrine energy balance controls of amphibians with that of other vertebrate taxa.

2. Materials and methods

2.1. Animals

S. hammondi egg clutches were collected in March 2003 in Riverside County, CA, under California scientific collecting permit no. 802003-01 issued to R.J.D. Tadpoles were raised in the laboratory in aquaria at 23 °C on a 12L:12D photoperiod. Tadpoles were fed a mixture of rabbit chow, agar, and gelatin molded into cubes (approximately 2×2×2 cm; see Rugh, 1962). Tadpoles were randomly chosen from six clutches and dispersed evenly among treatments. Following Etkin (1968), tadpoles classified as premetamorphic were of a developmental stage prior to significant hindlimb development (Gosner stage 31, 0.5–0.8 g body weight [g BW]; Gosner, 1960), and prometamorphic tadpoles were of developmental stages during hindlimb development (Gosner stage 36, 2.0–3.0 g BW). Juveniles used in the experiment were 9 months postmetamorphosis (2.0–4.0 g BW), and were from the same source of clutches as the tadpoles used in this study. Animal husbandry and use were conducted in accordance with the guidelines set by the Animal Care and Use Committee at the University of Michigan.

2.2. Food restriction experiments

2.2.1. Tadpole experiment

Tadpoles at each developmental stage were weighed and randomly assigned to food treatment (ad libitum food or no food) and distributed into three tanks (25×19×12.5 cm polystyrene cages) per treatment, each containing four tadpoles ($n=12/\text{treatment}/\text{developmental stage}$). On the fifth day of the experiment, tadpoles were collected at 12:00 h and euthanized by immersion in 0.005% (premetamorphic) or 0.01% (prometamorphic) benzocaine (doses were adjusted for body weight). At this time, wet body weight and Gosner stage were recorded, and brains were dissected and snap frozen for analysis of CRF peptide content by radioimmunoassay (RIA). Brains were dissected posterior to the optic tectum so that the telencephalon–diencephalon was analyzed. The remaining carcasses were snap frozen and stored at –20 °C for whole-body corticosterone extraction and RIA.

2.2.2. Juvenile experiment

Juveniles were housed in plastic shoeboxes with a 1-in. sandy soil layer at the bottom (two frogs per container). All animals were fed two pinhead crickets every day at 12:00 h for 7 days prior to the start of the experiment. Animals were stratified by body size and assigned to each treatment (daily fed or no food) such that there were no significant differences in body size between groups ($n=7/\text{treatment}$). At 12:00 h on the fifth day, animals were euthanized by immersion in 0.01% benzocaine. At this time blood was collected with heparinized capillary tubes via an incision in the suprabrachial artery and analyzed for corticosterone by

RIA. Brains were collected and snap frozen for analysis of CRF content; only the diencephalon was analyzed in juveniles.

2.3. Corticotropin-releasing factor RIA

The tissue extraction and CRF RIA procedure is described by Boorse and Denver (2004). Briefly, brain sections were weighed then extracted in boiling acetic acid prior to RIA. For premetamorphic tadpoles, two brain sections were pooled to yield enough tissue to detect CRF in the assay ($n=6$); for prometamorphic tadpoles and juveniles, individual brain sections were analyzed ($n=12$). Recoveries were determined by the addition of ^{125}I *Xenopus* CRF (xCRF) to tissue homogenates prior to extraction; recoveries averaged 71%. Boorse and Denver (2004) showed that acid extraction effectively removes or inactivates all CRF binding protein activity. The antiserum used in the RIA was produced in a rabbit against synthetic xCRF conjugated to human α -globulins (see Denver, 1997). The amino acid sequence of the mature *S. hammondi* CRF peptide is identical to the *Xenopus laevis* CRF (Boorse and Denver, 2003; Genbank accession no. AY262255); thus, the RIA is homologous for *S. hammondi* CRF. The cross-reactivity of the antiserum with other CRF-related peptides was minimal: 3% with *Xenopus* urocortin 1; no cross-reactivity with *Xenopus* urocortin 3 (Boorse and Denver, unpublished data). The RIA was conducted as described by Denver (1997). Intra- and interassay coefficients of variation were 5.2% and 16%, respectively. The mean minimum detectable limit of the assay (-2 S.D. of B_0) was 17 pg/tube. Dilutions of brain extracts exhibited parallelism to the standard curve in the RIA (Boorse and Denver, 2004). CRF content is expressed as picograms per milligram brain tissue.

2.4. Tissue extraction and corticosterone radioimmunoassay (RIA)

The tissue extraction procedure we used for analysis of corticosterone in whole tadpoles was described by Hayes and Wu (1995) and validated for *S. hammondi* tadpoles by Denver (1998). Briefly, tadpoles were weighed then homogenized in ethyl acetate. To estimate recoveries, 3500 cpm of [^3H]corticosterone was added to each homogenate. The extracts were fractionated by thin layer chromatography (TLC) to separate corticosterone from other lipids. The silica was extracted with diethyl ether, dried under nitrogen, and resuspended in RIA buffer (PBS-G; 0.02 M sodium phosphate, 0.6% saline, 10% gelatin; pH 7.3). For juveniles, 50–90 μL of plasma was assayed from each individual. [^3H]corticosterone (3,500 cpm) was added to each aliquot of plasma to estimate recoveries, plasma was extracted with diethyl ether, ether was dried under a stream of nitrogen, and extracts were resuspended in RIA buffer.

The corticosterone RIA was conducted as described by Licht et al. (1983). Anticorticosterone serum was purchased

from Esoterix Endocrinology (Calabasas, CA) and [^3H]corticosterone (70 Ci/mmol) from Perkin Elmer Life Science Products, Inc. (Boston, MA). Intra- and interassay coefficients of variation were 5% and 11.5%, respectively. The mean minimum detectable limit of the assay was 27 pg/tube. For tadpoles, whole-body CORT content is expressed as ng/g BW), and for juveniles, plasma CORT concentration is expressed as nanograms per milliliter.

2.5. Statistical analysis

Hormone data for tadpoles were analyzed using two-way analysis of variance (ANOVA), with CORT or CRF measurement as the dependent variable, and developmental stage (premetamorphic or prometamorphic) and food treatment (fed or food-deprived) as independent variables ($\alpha=0.05$). The interaction between developmental stage and food treatment was included in the model, but removed when not significant. Because CRF content and corticosterone concentrations were analyzed differently in tadpoles and juveniles, tadpole measures were statistically analyzed separately from juvenile measures. We used t tests to compare mean plasma corticosterone and diencephalon CRF content between fed and food-deprived juveniles. A nonparametric Mann–Whitney U test was used to determine differences in developmental stage between fed and unfed pre- and prometamorphic tadpoles ($\alpha=0.05$). All statistics were conducted in SAS v. 8.0 for Windows (SAS Institute, Cary, NC).

3. Results and discussion

3.1. Ontogenetic changes in the corticosterone response to food deprivation

As in many studies conducted in diverse vertebrate taxa, we used a food deprivation paradigm to determine if components of the HPI axis respond to energy balance signals, and if this response changes with development in *S. hammondi*. In juvenile or adult birds and mammals, plasma glucocorticoid concentration increases with food deprivation (Dallman et al., 1999; Lynn et al., 2003). This “stress” response is thought to promote the mobilization of energy reserves and foraging behavior given the high demand for resources to maintain metabolism and thermogenesis in endotherms (Sapolsky et al., 2000). By contrast, we found that plasma corticosterone concentration was significantly lower in food-deprived *S. hammondi* juveniles compared with fed animals (t test, $df=13$, $P=0.049$; Fig. 1). Previously, we found that juvenile *X. laevis* maintains relatively constant plasma corticosterone concentrations through 31 days of food deprivation (Crespi and Denver, 2004a). Thus, in both species, food deprivation does not activate the HPI axis in juvenile frogs. Since increased plasma corticosterone concentration is associated with the breakdown of fat stores

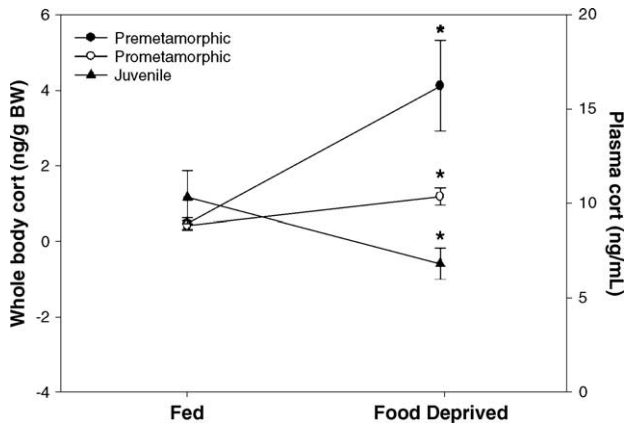


Fig. 1. Reaction norm representation of corticosterone responses to 5 days of food deprivation among three developmental stages of *S. hammondi*. Data reflect the mean \pm S.E.M. of whole-body corticosterone content of premetamorphic (Gosner stage 31) and prometamorphic (Gosner stage 36) tadpoles (left axis); plasma corticosterone concentration was assayed in juveniles (right axis). Asterisks indicate significant differences between food groups within each stage (t test, $\alpha < 0.05$).

and gluconeogenesis (Sapolsky et al., 2000), these results are consistent with the idea that frogs enter a state of energy conservation when food availability is low (Pough, 1985; Storey, 2002).

The contrasting responses of *X. laevis* and *S. hammondi* may reflect interspecific differences among anurans in the physiological response to fasting. The decrease in plasma corticosterone in *S. hammondi*, a desert-adapted species that typically endures long periods without food, may be part of a suite of physiological adjustments that reduce energy expenditure in a state of food deprivation. Previous studies have shown that 2 weeks of food deprivation caused a 50% reduction in oxygen consumption and a decrease in liver enzyme activity in *S. hammondi* and *S. couchii* (Seymore, 1973; Storey, 2002). Metabolic depression is a common response to adverse environmental conditions and underlies organismic responses to prolonged adverse conditions (e.g., hibernation, torpor, and estivation; Pinder et al., 1992; Storey, 2002). Starvation-induced reduction in circulating corticosterone may slow the utilization of stored fuels, and because corticosterone is known to have osmoregulatory functions (McCormick, 2001), this reduction may be a physiological adaptation to a xeric environment. Because *X. laevis* is an aquatic frog that evolved in more temperate climates, there may not have been selection

pressure to evolve physiological mechanisms to reduce metabolic rate below its resting state during times of food deprivation.

To determine if the HPI axis responds to food deprivation in larval life stages, we also analyzed corticosterone in fed and food-deprived premetamorphic and prometamorphic *S. hammondi* tadpoles. Because tadpoles forage more actively, have higher metabolic rates, and have faster growth rates than juvenile frogs (Burggren and Just, 1992), we predicted that the HPI axis in *S. hammondi* tadpoles would be stimulated by food deprivation. As predicted, both stages of *S. hammondi* tadpoles increased whole-body corticosterone content after 5 days of food deprivation (ANOVA model: $F_{(3,31)}=12.85$, $P < 0.0001$; food treatment: $F=20.56$, $P < 0.0001$; Fig. 1). We previously showed that 4 days of food restriction (i.e., a reduced diet sufficient for maintenance metabolism but not for growth) increased whole-body corticosterone content in premetamorphic leopard frog (*Rana pipiens*) tadpoles (Glennemeier and Denver, 2002b). However, the current study is the first to compare the HPI axis response to food restriction among different developmental stages of the same amphibian species to show that the response varies with life cycle stage.

Interestingly, premetamorphic tadpoles exhibited a significantly greater corticosterone response than prometamorphic tadpoles (ANOVA treatment \times developmental stage interaction: $F=10.25$, $P=0.003$; Fig. 1). This increase in corticosterone in tadpoles was associated with significant reductions in both growth and development rate (Table 1), and is likely involved in the mobilization of stored fuels required for maintenance metabolism as observed in birds and mammals (Sapolsky et al., 2000; Lynn et al., 2003). The increased sensitivity of the HPI axis of premetamorphic tadpoles to nutritional stress is consistent with our previous findings that *R. pipiens* and *X. laevis* premetamorphic tadpoles exhibit greater corticosterone responses to shaking/confinement stress and adrenocorticotrophic hormone injection than prometamorphic tadpoles (Glennemeier and Denver, 2002a). The greater corticosterone content in premetamorphic tadpoles corresponds to a greater loss (32%) of body weight during fasting compared with prometamorphic tadpoles (10%; Table 1). The difference in stored fat between the two tadpole stages could be causally related to the differences in the HPI response to fasting. Unlike premetamorphic tadpoles, prometamorphic tadpoles had visible fat bodies at the end of the experiment

Table 1

Effects of 5-day fasting on body weight (mean \pm S.E.M.) and Gosner stage (mode with proportion of individuals of that stage in parentheses) in premetamorphic (Gosner stage 31) and prometamorphic (Gosner stage 36) *S. hammondi* tadpoles

| Treatment | Premetamorphic | | | Prometamorphic | | |
|---------------|--------------------|------------------|------------|--------------------|------------------|------------|
| | Initial weight (g) | End weight (g)* | End stage* | Initial weight (g) | End weight (g)* | End stage* |
| Fed | 0.63 \pm 0.018 | 1.30 \pm 0.127 | 34 (9/12) | 2.28 \pm 0.068 | 4.12 \pm 0.274 | 40 (8/12) |
| Food-deprived | 0.62 \pm 0.023 | 0.42 \pm 0.033 | 31 (12/12) | 2.33 \pm 0.051 | 2.10 \pm 0.090 | 36 (10/12) |

* $P < 0.0001$.

(E.J. Crespi, unpublished data), suggesting that these tadpoles were able to mobilize fuels from lipid stores for maintenance metabolism.

The effect of corticosterone on development also differs between tadpole stages, such that corticosterone slows development in premetamorphic tadpoles (Hayes et al., 1993; Glennemeier and Denver, 2002c) but synergizes with thyroid hormone to accelerate metamorphosis in prometamorphic tadpoles (Kikuyama et al., 1993; Hayes, 1997; Denver, 1997; Krain and Denver, 2004). Resource availability is one of several environmental factors known to accelerate metamorphosis of prometamorphic tadpoles of *S. hammondi* and other anuran species (Denver et al., 1998; Morey and Reznick, 2000; Boorse and Denver, 2003). The results from this study, showing for the first time that the HPI axis is activated by fasting in prometamorphic tadpoles, could be a physiological link between resource availability and the timing of metamorphosis.

In the current study, we did not observe an acceleration of development in prometamorphic tadpoles over the 5-day period of food deprivation despite an increase in corticosterone content (Table 1). One possible explanation for this result is that the developmental response to food deprivation in prometamorphic tadpoles is condition-dependent: if tadpoles were in good condition (e.g., ample stored fat) in our experiment, the 5-day period of fasting may have been insufficient to observe an increase in development rate. The prometamorphic tadpoles in the current study weighed more for their stage of development than those used in previous studies where we observed accelerated metamorphosis with fasting (Denver et al., 1998; Boorse and Denver, 2003), suggesting that these tadpoles were in a better nutritional state than those in the previous studies. In other vertebrates, animals with greater fat stores exhibit weaker corticosterone responses to stressors (Kitaysky et al., 1999; O'Reilly and Wingfield, 2003) possibly through suppression of the HPA axis by factors secreted by adipocytes (Ahima et al., 1996; Katoh et al., 2004). Thus, while stress hormones likely mediate the translation of energetic status/growth opportunity information into a developmental response, fat stores may provide energy balance cues that modulate the strength of HPI axis activation in conditions of low food availability.

The magnitude of the developmental response of prometamorphic tadpoles also varies with the type of environmental stressor. For example, simulated pond drying, a common environmental stressor in *S. hammondi* populations (Morey and Reznick, 2004), accelerated development and increased thyroid hormone content by 24 h, but food deprivation did not affect either of these parameters until 4 days (Denver et al., 1998; Boorse and Denver, 2003). The magnitudes of the corticosterone responses to these stressors follow a similar pattern: water volume reduction caused a sevenfold increase in whole-body corticosterone content (Denver, 1998), while 5 days of food deprivation caused only a threefold increase in corticosterone in the current study. The sensitivity of the HPI axis to different

stressors may ultimately be related to the survival threat presented by each stressor (Boorse and Denver, 2003). Desiccation resulting from pond drying poses an immediate threat to tadpole survival (Newman, 1992), while the negative consequences of reduced resource availability could take days or weeks to be expressed depending on the amount of energy the tadpole has stored in fat bodies.

3.2. Ontogenetic changes in CRF response to food deprivation

The expression of CRF in specific brain nuclei/regions is known to be altered by food intake/energy balance in mammals (Heinrichs and Richard, 1999). Our current analysis of brain CRF peptide content in tadpoles and juvenile toads showed that CRF content increases with development, as shown in previous studies (Denver, 1997; Carr and Norris, 1990), and tends to increase in response to food deprivation in tadpoles but not in juveniles (Fig. 2). In tadpoles, forebrain/diencephalon CRF peptide content was significantly higher during prometamorphosis than premetamorphosis, but the elevation between starved and fed individuals was marginally nonsignificant (ANOVA model: $F_{(2,33)}=16.76$, $P<0.0001$; stage: $F=29.47$, $P<0.0001$; food treatment: $F=4.05$, $P=0.052$; Fig. 2). In juvenile toads, mean diencephalon CRF peptide content was higher than in the combined telencephalon/diencephalon section analyzed in the tadpole stages. However, diencephalon CRF content did not differ between fed and food-deprived juveniles (135 ± 13 pg/mg and 134 ± 21 pg/mg, respectively).

The increase in CRF in *S. hammondi* with developmental stage reflects the maturation of the neurosecretory cells in the diencephalon that occurs during prometamorphosis

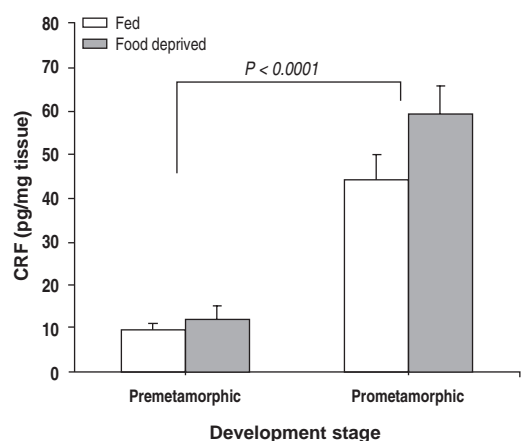


Fig. 2. Effect of 5 days of food deprivation on CRF content in forebrain/diencephalon sections of pre- and prometamorphic *S. hammondi* tadpoles. Bars indicate mean \pm S.E.M. for each treatment group. For premetamorphic tadpoles two individual brain sections were pooled in each sample ($n=6$); for prometamorphic tadpoles each sample consisted of an individual brain section ($n=12$). CRF content in prometamorphic tadpoles was significantly higher than in premetamorphic tadpoles (ANOVA stage effect P value shown), and CRF content tended to increase with food deprivation, although this difference was not statistically significant within either stage.

(reviewed by Denver, 1996). It may seem contradictory that premetamorphic tadpoles have lower CRF content compared to prometamorphic tadpoles, yet they show greater corticosterone content in response to food deprivation than prometamorphic tadpoles. The greater corticosterone response in premetamorphic tadpoles could result from immature negative feedback by glucocorticoids at this stage of development (i.e., glucocorticoid receptors may not yet be expressed on CRF neurons). Alternatively, other factors are known to stimulate ACTH in vertebrates (e.g., arginine vasopressin), and these factors may play a larger role in stimulating corticosterone secretion in premetamorphic tadpoles when neuroendocrine axes are at the beginning stages of their development. However, little is known about the action of these factors in premetamorphic tadpoles at this time.

The trend towards an elevation of CRF content with food deprivation in tadpoles is consistent with the elevation in corticosterone measured in these animals (see above). It should be noted that measures of CRF content in large brain regions provide only a gross analysis of CRF neuronal function. The tissues that we analyzed contain discrete collections of CRF neurons that are likely to be differentially influenced by energy status; therefore the effect of food deprivation on CRF content in the entire brain section may have been too weak to detect with our small sample sizes. Future studies using histochemistry are required to analyze these different cell populations.

Earlier we found that preoptic area CRF peptide content increased in prometamorphic tadpoles exposed to water volume reduction (Denver, 1997) and in juvenile frogs subjected to shaking/confinement stress (Boorse and Denver, 2003; Yao et al., 2004). The current study is the first to suggest that CRF peptide content increases in response to nutritional stress in an amphibian, indicating that CRF neurons are influenced by indicators of energy balance. Currently, the amphibian counterparts of the identified energy balance indicators/adiposity signals in mammals are unknown. Leptin, a hormone secreted by adipose cells in proportion to the amount of stored fat has been shown to mediate food intake behavior in mammals via interactions with CRF neurons (Zhang et al., 1994; Uehara et al., 1998). However, a similar molecule has yet to be identified in any ectothermic vertebrate. Other factors known to function as energy balance indicators in mammals and fishes, such as glucose, insulin, glucagon-like peptide-1, neuropeptide-Y, or gut-secreted neuropeptides (MacKenzie et al., 1998; Kalra et al., 1999), could also function as energy balance indicators in amphibians and require further study.

3.3. Ontogeny of CRF and corticosterone regulation of food intake

We and others have shown that CRF and corticosterone modulate energy balance by affecting food intake across developmental stages in amphibians. The mammalian model of hypothalamic control of food intake (e.g., appetite) involves the counterregulation of appetite-suppressing and

appetite-stimulating neuropeptides (Kalra et al., 1999), and recent studies suggest that this model also applies to amphibians. Two of the most potent inhibitors of food intake in mammals, CRF-like peptides and the proopiomelanocortin-derived peptide α -melanocyte-stimulating hormone (α -MSH), also suppress food intake and prey-catching behaviors in frogs (CRF: Corpas et al., 1991; Carr et al., 2002; Crespi and Denver, 2004a,b; α -MSH: Carr et al., 2002). The anorexigenic response to exogenous CRF is seen in premetamorphic tadpoles (Fig. 3), thus showing that CRF receptors are expressed at this early stage of development.

In the context of the stress response, these experiments suggest that CRF, in addition to its hypophysiotropic actions, also has effects on behavior consistent with its proposed role as a neurotransmitter/neuromodulator. We found that, in addition to the inhibitory effect of CRF on food intake, i.c.v. injection of CRF stimulated locomotion (e.g., swimming) in both pre- and prometamorphic tadpoles (Fig. 3). This coordinated behavioral response to exogenous CRF mirrors behaviors exhibited during the “flight or fight” response. In contrast to the effects of CRF on locomotion in tadpoles, we found that i.c.v. injection of CRF inhibited prey-catching behavior in juvenile toads, while injection of the CRF receptor antagonist α -helical CRF_(9–41) increased movement associated with prey catching (Fig. 4). The anorexigenic effect of CRF (like α -MSH) in juveniles may result from the inhibition of oculomotor activity associated with prey detection, and as suggested by Carr et al. (2002), and may be associated with the expression of stress-induced cryptic behaviors in toads (versus the flight response in tadpoles). The behavioral differences between tadpoles and juvenile/adult in their response to CRF injection also may reflect differences in feeding ecology between the two life stages: tadpoles are active foragers, while juveniles/adults are sit-and-wait predators.

In addition to stress-induced anorexia, we found that endogenous CRF modulates appetite in the absence of stress in amphibians (Crespi and Denver, 2004a,b). By blocking CRF receptors with i.c.v. injection of α -helical CRF_(9–41) we observed an increase in meal size in juveniles of both *X. laevis* (Crespi and Denver, 2004a) and *S. hammondi* (Fig. 4), suggesting that endogenous CRF in the unstressed state exerts a suppressive effect on food intake. Injection of α -helical CRF_(9–41) also stimulated foraging behavior in prometamorphic tadpoles, but not in premetamorphic tadpoles (Fig. 3). By contrast, α -helical CRF_(9–41) injection significantly reduced swimming (i.e., increased resting) in premetamorphic tadpoles, suggesting that endogenous CRF regulates locomotor behavior at this stage. Feeding behavior may have been unaffected by α -helical CRF_(9–41) in premetamorphic tadpoles because CRF neurons controlling foraging behavior have not yet developed to their full biosynthetic capacity during these early stages (Carr and Norris, 1990; see Denver, 1996) (i.e., receptors are present before endogenous ligand is secreted). Alternatively, the neural circuitry controlling food intake

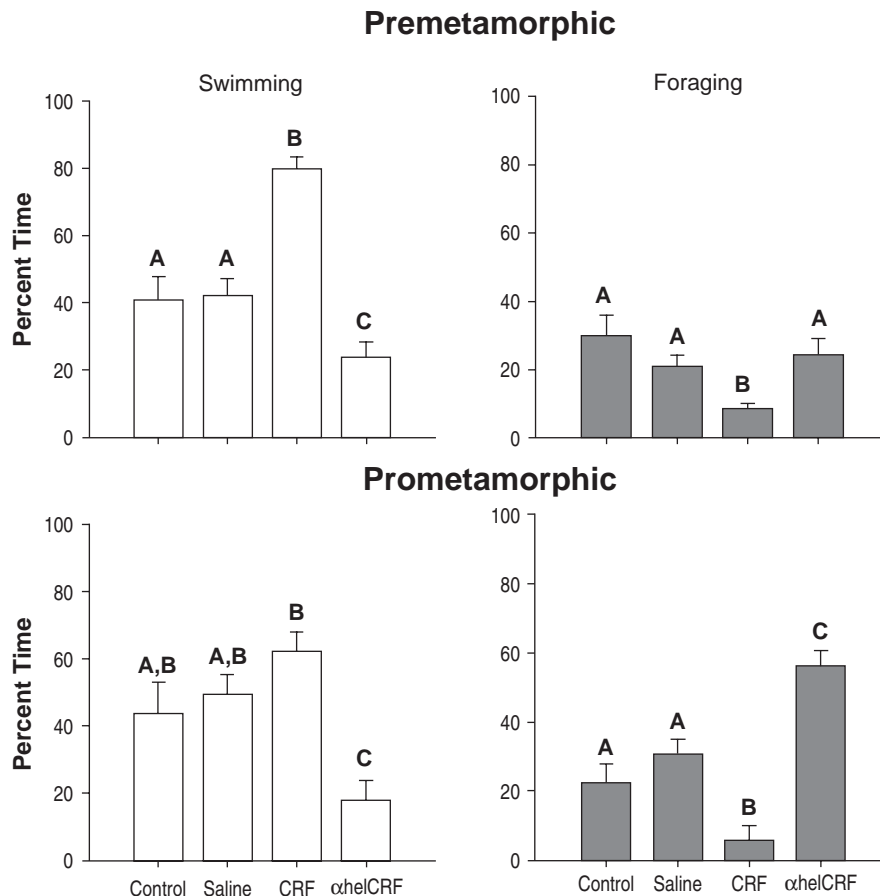


Fig. 3. Effects of i.c.v. injection of CRF and α -helical CRF₍₉₋₄₁₎ on swimming and foraging behavior in pre- and prometamorphic *S. hammondi* tadpoles. Behavioral observations were made for 2 min on each individual ($n=6$) approximately 1–2 h after injection; data are represented as the mean \pm S.E.M. percent time tadpoles were observed performing the behavior. Letters indicate statistical differences between treatment groups as determined by Duncan's multiple comparisons tests ($P<0.05$). Data are from Crespi and Denver (2004b).

may not be fully developed until prometamorphic stages. In mammals, hypothalamic controls of food intake do not fully develop until weaning begins (Grove and Smith, 2003). In each case the actions of neuroendocrine satiety signals develop after a phase of rapid growth, presumably to allow for maximum food intake (Ross et al., 2003). In an evolutionary context, this comparison suggests that the developmental timing of similar neuroendocrine feeding controls is selected according to the life history of each species.

By contrast to the anorexigenic effects of CRF, short-term corticosterone treatment stimulated food intake in both prometamorphic tadpoles and postmetamorphic amphibians (Crespi and Denver, 2004a,b), as has been shown in mammals and fishes (Dallman et al., 1999; Bernier et al., 2004). In addition, we showed that the subsequent increase in whole-body corticosterone that occurred several hours after i.c.v. CRF injection in prometamorphic *S. hammondi* tadpoles is responsible for the increased appetite at this time (Crespi and Denver, 2004b). These results suggest that activation of the HPI axis in response to a stressor maintains energy balance homeostasis through the opposing, and

temporally distinct, effects of CRF and corticosterone on food intake (see Sapolsky et al., 2000).

In mammals and fishes, the appetite-stimulating effects of corticosterone may be mediated, at least in part, by hypothalamic neuropeptide-Y (NPY) neurons (Stanley et al., 1989; Silverstein and Plisetskaya, 2000; Bernier et al., 2004). NPY is an evolutionarily conserved orexigenic peptide, and although we do not yet know if a functional relationship exists between NPY and corticosterone in amphibians, we have shown that NPY has orexigenic effects in *X. laevis* juveniles (Crespi and Denver, 2004a). The conserved actions of corticosterone and NPY on appetite suggest that there could be regulatory pathways that link corticosterone to NPY in amphibians similar to other vertebrates.

The conserved effects of CRF and corticosterone, as well other neuropeptides, on food intake suggest that feeding regulatory pathways are evolutionarily conserved. Studies in mammals have shown that other components of the CRF system, such as CRF receptors (Makino et al., 1998; Pellemounter et al., 2002) and CRF-binding protein (Burrows et al., 1998; Karolyi et al., 1999), play important roles in

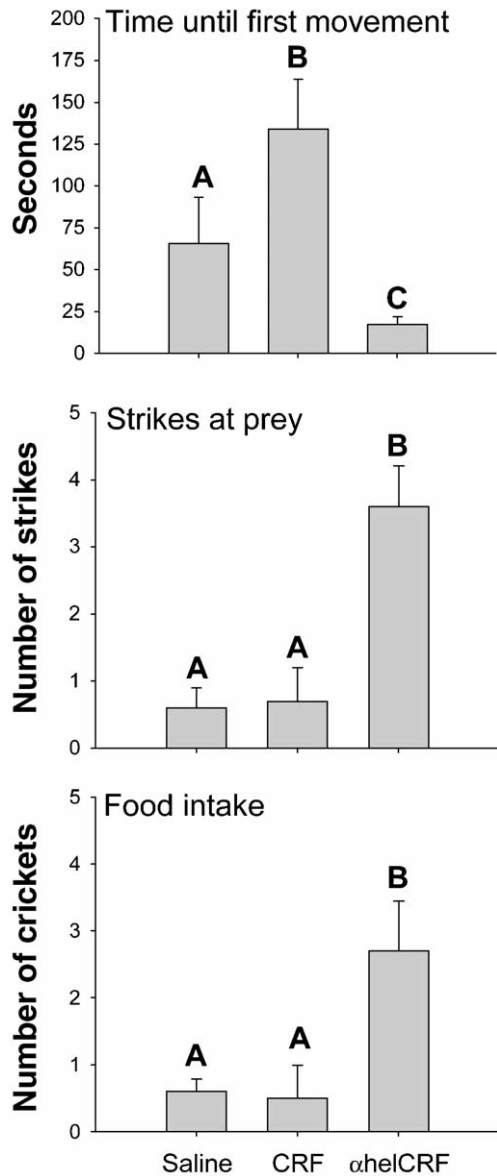


Fig. 4. Effects of i.c.v. injection of CRF and α -helical CRF on prey-catching behavior in *S. hammondi* juveniles. Toads were placed in plastic shoebox lined with live pinhead crickets and observed for 3 min ($n=7$). Toads were fed 4 h before the experiment to minimize hunger, and maximize the ability to detect an appetite-stimulating effect. Bars indicate mean \pm S.E.M. for each variable; letters indicate statistical differences between treatment groups as determined by Duncan's multiple comparisons tests ($P<0.05$). Data are from Crespi and Denver (2004b).

food intake regulation by either mediating CRF actions or modulating CRF activity. Further study in amphibians and other nonmammalian vertebrates is needed to determine if similar components of the CRF system also play central roles in the regulation of food intake and energy balance.

3.4. Summary and conclusions

Our studies in the Western spadefoot toad show that CRF and corticosterone affect energy balance and food intake

across developmental stages in amphibians, but the specific actions may have been adapted to the metabolic demands and ecological conditions unique to each life history stage. We found that food deprivation activated the HPI axis in *S. hammondi* tadpoles, but depressed HPI activity in juveniles. The contrasting responses may be physiological adaptations to the biphasic life cycle, with further modification through adaptation to a xeric environment: spadefoot toad tadpoles are adapted for rapid growth that allows for metamorphosis before the disappearance of a rapidly drying pond, while fossorial juvenile and adult toads must conserve resources to survive for periods of fasting that could last for months to years (Newman and Dunham, 1994). Furthermore, the experiments we and others have conducted clearly show that CRF has anorexigenic actions, but corticosterone has orexigenic actions in both larval and juvenile amphibians, as shown in other vertebrates. Our findings suggest that CRF and corticosterone play important roles in the regulation of stress-induced changes in feeding behavior and, during later developmental stages, in the regulation of daily food intake in amphibians.

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