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Stress hormones mediate developmental plasticity in vertebrates with complex life cycles

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ABSTRACT

The environment experienced by developing organisms can shape the timing and character of developmental processes, generating different phenotypes from the same genotype, each with different probabilities of survival and performance as adults. Chordates have two basic modes of development, indirect and direct. Species with indirect development, which includes most fishes and amphibians, have a complex life cycle with a free-swimming larva that is typically a growth stage, followed by a metamorphosis into the adult form. Species with direct development, which is an evolutionarily derived developmental mode, develop directly from embryo to the juvenile without an intervening larval stage. Among the best studied species with complex life cycles are the amphibians, especially the anurans (frogs and toads). Amphibian tadpoles are exposed to diverse biotic and abiotic factors in their developmental habitat. They have extensive capacity for developmental plasticity, which can lead to the expression of different, adaptive morphologies as tadpoles (polyphenism), variation in the timing of and size at metamorphosis, and carry-over effects on the phenotype of the juvenile/adult. The neuroendocrine stress axis plays a pivotal role in mediating environmental effects on amphibian development. Before initiating metamorphosis, if tadpoles are exposed to predators they upregulate production of the stress hormone corticosterone (CORT), which acts directly on the tail to cause it to grow, thereby increasing escape performance. When tadpoles reach a minimum body size to initiate metamorphosis they can vary the timing of transformation in relation to growth opportunity or mortality risk in the larval habitat. They do this by modulating the production of thyroid hormone (TH), the primary inducer of metamorphosis, and CORT, which synergizes with TH to promote tissue transformation. Hypophysiotropic neurons that release the stress neurohormone corticotropin-releasing factor (CRF) are activated in response to environmental stress (e.g., pond drying, food restriction, etc.), and CRF accelerates metamorphosis by directly inducing secretion of pituitary thyrotropin and corticotropin, thereby increasing secretion of TH and CORT. Although activation of the neuroendocrine stress axis promotes immediate survival in a deteriorating larval habitat, costs may be incurred such as reduced tadpole growth and size at metamorphosis. Small size at transformation can impair performance of the adult, reducing probability of survival in the terrestrial habitat, or fecundity. Furthermore, elevations in CORT in the tadpole caused by environmental stressors cause long term, stable changes in neuroendocrine function, behavior and physiology of the adult, which can affect fitness. Comparative studies show that the roles of stress hormones in developmental plasticity are conserved across vertebrate taxa including humans.

1. Introduction

The environment experienced by an organism during development can have profound effects on phenotypic expression. The interaction between the genotype and the environment to generate different phenotypes is often referred to as phenotypic plasticity or developmental plasticity (Gilbert, 2016; Moczek et al., 2011; Sultan, 2019; West-Eberhard, 2005a). Here I will use the term developmental plasticity to distinguish environmental effects on phenotypic expression occurring during postembryonic development from those that occur later in life. Another term found primarily in the biomedical literature is developmental programming, which is often used to describe adverse effects of early life experience (fetal, neonatal, early postnatal) on adult (or juvenile) disease development (see the Developmental Origins of Disease Hypothesis) (Ozanne and Costancia, 2007). Developmental plasticity can generate adaptive changes in morphology, physiology and behavior,

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it can be shaped by natural selection, and it can be a driving force in phenotypic evolution (Lafuente and Beldade, 2019; Levis and Pfennig, 2019, 2020; West-Eberhard, 2003, 2005a).

There are two modes of development in vertebrates, indirect and direct, with the major difference being the presence of a larval stage in indirect development. Amniotes (reptiles, birds and mammals) all have direct development, while most extant fish and amphibian species have indirect development (Laudet, 2011). Animals with indirect development display a complex life cycle, where the animal begins life as a larva, then undergoes a metamorphosis to the juvenile. The larval period is typically a growth life history stage, and larvae often exploit different ecological niches from adults, thus avoiding competition for resources. This ability to change form and function across life stages allows for body forms that specialize in growth, dispersion, or reproduction when those fitness components are concentrated in a particular life stage (Moran, 1994). The ancestral chordate mode of development was likely indirect, and direct development evolved multiple times in different lineages (Hall and Wake, 1999; Laudet, 2011).

Animal embryonic development tends to be canalized, and buffered from environmental influences, while postembryonic development can be strongly influenced by the environment (Gibson and Wagner, 2000; Gilbert, 2012). In direct developing species, both embryonic and postembryonic (e.g., fetal) development occurs within the egg or *in utero*. By contrast, in animals with complex life cycles, while embryonic development also occurs in the egg, postembryonic development occurs during the larval stage. Since larvae are typically free swimming, and thus exposed to a range of environmental factors, postembryonic development in species with complex life cycles is often impacted by environmental factors in more direct ways than in species with direct development (but, see effects of temperature on sex determination in reptiles) (Matsumoto and Crews, 2012). Developmental plasticity is widespread in animals with complex life cycles, and it can have adaptive roles, affecting growth and survival during the larval stage, and performance and reproductive success after metamorphosis (a.k.a., carry-over effects).

Cephalochordates (i.e., *Amphioxus*), urochordates (i.e., *Ciona*), cyclostome vertebrates (i.e., lampreys), most teleost fishes and most amphibians have complex life cycles (Laudet, 2011). Among chordates with complex life cycles, the best studied are the anuran amphibians (frogs and toads). The larvae of anurans (tadpoles) are aquatic and specialized for growth. Upon reaching a minimum body size they become competent to undergo morphological, biochemical and physiological transformation (metamorphosis) into the terrestrial or semi-terrestrial juvenile frog. The environmental conditions experienced during the tadpole stage, such as food level, population density, pond duration and predation risk can affect tadpole behavior and morphology, the timing of and body size at metamorphosis, and adult phenotypic expression (Denver et al., 1998; Goater, 1994; Newman, 1992; Relyea, 2007; Werner, 1986; Wilbur and Collins, 1973).

The postembryonic period corresponds with the development of the circulatory system, and so it is at this stage of development when signaling via the endocrine system becomes important. Hormones, through their regulation of gene expression and cell physiology have critical roles in animal development. Thyroid hormone (TH), whose secretion is controlled by pituitary thyrotropin (TSH), is the primary inducer of amphibian metamorphosis (Brown and Cai, 2007). In tadpoles, the central regulation of the production of TH and its actions in peripheral tissues depend on hormones of the neuroendocrine stress axis (the hypothalamo-pituitary-adrenal/interrenal, or HPA/HPI axis; the amphibian interrenal gland is homologous to the mammalian adrenal cortex). Here I use the general term 'stress hormone' to refer to hormones that are produced and act within the HPA/HPI axis. These include neurohormones such as corticotropin-releasing factor (CRF) and related peptides, pituitary corticotropin (ACTH) and glucocorticoids (GCs) produced by adrenal cortical (or interrenal) cells (e.g., cortisol and corticosterone - CORT). There is strong support for CRF playing a pivotal role in life history transitions in vertebrates (Denver, 2009b, 2017; Denver and Middlemis-Maher, 2010; Watanabe et al., 2016). Research conducted over the past thirty years has shown that stress hormones have central roles in mediating environmental effects on developmental plasticity in amphibians and other vertebrates (Boorse and Denver, 2006; Denver, 2009c; Yao and Denver, 2007). In this review I discuss the neuroendocrine mechanisms that drive developmentally plastic responses to environmental change with a focus on amphibian species.

2. Neuroendocrine control of amphibian metamorphosis

Stress hormones have profound effects on postembryonic development, influencing survival of larvae and subsequent physiological, morphological and behavioral traits of adults (Alyamani and Murgatroyd, 2018; Denver, 1997c, 2017; Denver and Middlemis-Maher, 2010; Fogelman and Canli, 2019; Liu and Nusslock, 2018; Thayer et al., 2018). Below I describe the major components of vertebrate HPA/HPI and hypothalamo-pituitary-thyroid (HPT) axes, then in the following section I discuss the roles that stress hormones play in mediating environmental effects on amphibian development, and in programming the phenotype of the adult frog. I use the terms first coined by Etkin (1968) to describe the different stages of anuran metamorphosis (Fig. 1A): 'pre-metamorphosis', when the larvae grows but little or no morphological change occurs and plasma TH concentration is low; 'prometamorphosis', when hindlimb growth accelerates and plasma TH concentration rises; and 'metamorphic climax', the final and most rapid phase of morphological change when thyroid activity is at its peak.

2.1. Thyroid hormone

In non-mammalian species TH production is controlled by neurohormones typically associated with the HPA/HPI axis. Also, TH influences HPA/HPI axis function, and CSs modulate TH actions at target tissues. Thyroid hormone orchestrates the suite of cellular, molecular, biochemical and morphological changes that occur during tadpole metamorphosis, acting via nuclear receptors (TH receptors – TRs) that belong to the steroid hormone receptor superfamily to induce or repress genes whose protein products underlie tissue transformations (Buchholz, 2017; Kyono et al., 2016; Mangelsdorf et al., 1995). Thyroid hormone enters cells via membrane-resident transporters, is transported within the cell by cytosolic binding proteins, and is metabolized to active and inactive forms by intracellular monodeiodinase enzymes (Choi et al., 2015; Denver, 2013).

The hypothalamus produces neurohormones that control TSH secretion by the anterior pituitary gland. In mammals, the tripeptide amide thyrotropin-releasing hormone (TRH) is the primary neurohormone controlling TSH release. By contrast, TRH is inactive on tadpole TSH secretion, although the *trh* gene is expressed in the brain and pituitary of amphibians (Denver, 1996; Denver and Licht, 1989; Kikuyama et al., 1993; Manzon and Denver, 2004; Norris and Dent, 1989; Okada et al., 2004) and TRH can stimulate TSH release from adult frog pituitary glands (Denver, 2009a; Galas et al., 2009). Instead, TRH appears to be primarily a prolactin releasing factor in tadpoles (Denver, 2017; Kikuyama et al., 2019). Many studies now support that the secretion of TSH by the tadpole pituitary gland is under stimulatory control by CRF and related peptides (Fig. 1B).

2.2. Corticotropin-releasing factor and related peptides

Corticotropin-releasing factor and related peptides regulate neuroendocrine, autonomic and behavioral responses to physical and emotional stress (Aguilera, 1998; Deussing and Chen, 2018; Yao and Denver, 2007), and play central roles in developmental plasticity in vertebrates (Denver, 2009b, 2017; Denver and Middlemis-Maher, 2010; Watanabe et al., 2016). Corticotropin-releasing factor is a 41 amino acid polypeptide that was named for its role in inducing pituitary ACTH

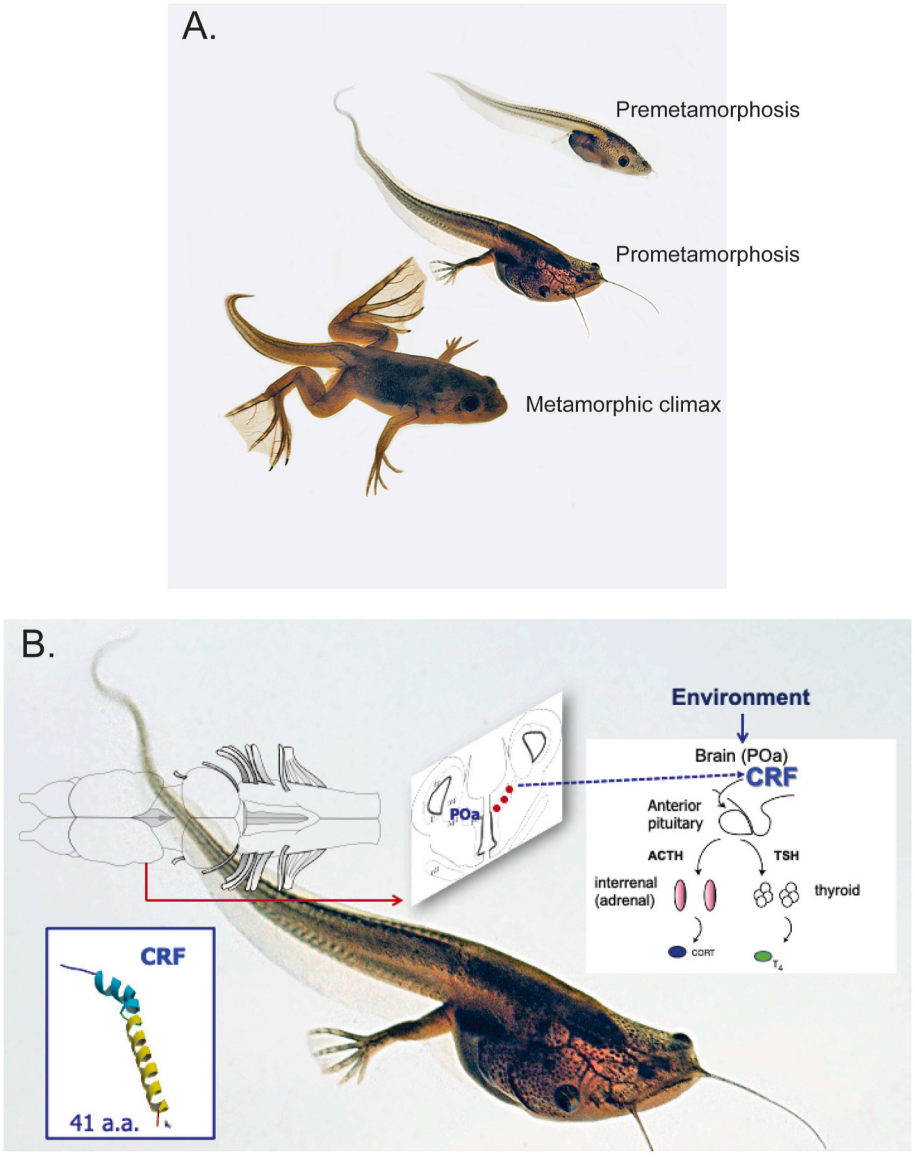


Fig. 1. Amphibian metamorphosis. A. Shown are the broad stages of tadpole (*Xenopus laevis*) metamorphosis using the terms coined by Etkin (Etkin, 1968). **B.** Corticotropin-releasing factor (CRF) is a thyrotropin (TSH)-releasing factor in tadpoles and other nonmammalian species. It is also a corticotropin (ACTH)-releasing factor in these species, although arginine vasotocin has a more prominent role. Neurosecretory CRF neuron cell bodies located in the anterior preoptic area (POa) respond to environmental stressors, releasing their contents into the pituitary portal system to control thyroid and interrenal (amphibian homolog of the mammalian adrenal cortex) activity. The *Xenopus* neuroanatomical diagrams are from Tuinhof and colleagues (Tuinhof et al., 1998). Tadpole photos by David Bay.

secretion (Turnbull and Rivier, 1997; Vale et al., 1997; Vale et al., 1981). It is a member of a family of related peptides in vertebrates that includes the fish urotensins-I, frog sauvagine and the urocortins (urocortins 1–3) (Boorse et al., 2005; Boorse and Denver, 2006; Dautzenberg and Hauger, 2002; Deussing and Chen, 2018). Tetrapods have four paralogous lineages of CRF-like peptides that arose before the divergence of the actinopterygian and sarcopterygian fishes (Boorse et al., 2005; Lovejoy and Balment, 1999). These peptides share a deep evolutionary relationship with invertebrate diuretic peptides (Lovejoy and Balment, 1999).

2.3. Actions of CRF peptides

Corticotropin-releasing factor and related peptides act on cells via two G protein-coupled receptors, designated CRF_1 and CRF_2, and their actions can be modulated by a secreted binding protein (CRF-BP) whose major function appears to be to modulate access of CRF peptides to CRF receptors (Dautzenberg and Hauger, 2002; Deussing and Chen, 2018; Seasholtz et al., 2001). Orthologs of mammalian CRF_1 and CRF_2 have been isolated from other vertebrate species including frogs (Dautzenberg et al., 1997; Ito et al., 2006). The two receptors exhibit distinct expression patterns, and mediate the actions of CRF peptides in the central nervous system and also in peripheral tissues (Boorse and Denver, 2006; Deussing and Chen, 2018). Corticotropin-releasing factor and urocortin 1 activate both CRF_1 and CRF_2, but CRF has higher affinity for CRF_1, and urocortin 1 (and sauvagine) has higher affinity for CRF_2 (Boorse et al., 2005; Dautzenberg et al., 1997; Dautzenberg and Hauger, 2002); urocortins 2 and 3 are selective for CRF_2 (Hauger et al., 2003). The CRF_1 is expressed on corticotropes and transduces CRF actions on ACTH secretion (Deussing and Chen, 2018; Yao and Denver, 2007), and ACTH induces CS secretion by the adrenal cortex/interrenal glands. In both mammals and frogs, CRF acts synergistically with the nonapeptide arginine vasopressin (AVP; arginine vasotocin – AVT, is the amphibian hormone) to induce ACTH secretion, and AVT appears to be the principal ACTH releasing factor in amphibians (Kikuyama et al., 2019). In amphibians and birds the CRF_2 is expressed in pituitary thyrotropes where it mediates the actions of CRF peptides on TSH secretion (see below) (Okada et al., 2009; Watanabe et al., 2016).

In addition to their central roles in regulating pituitary hormone secretion, CRF and related peptides are expressed throughout the central nervous system of vertebrates, functioning as both neurotransmitters and neuromodulators, thereby coordinating behavioral and autonomic responses to stressors (Boorse and Denver, 2006; Deussing and Chen, 2018). Corticotropin releasing factor peptides have pivotal roles in the regulation of food intake (Crespi and Denver, 2005; Morimoto et al.,

2011), behavioral responses to stress (Bale and Vale, 2004; Sapolsky et al., 2000), and learning and memory consolidation (Fenoglio et al., 2006; Gulpinar and Yegen, 2004; Roozendaal et al., 2008; Todorovic et al., 2007). These peptides, their receptors and binding protein are also expressed in peripheral tissues where they have diverse physiological functions (Boorse and Denver, 2006; Deussing and Chen, 2018).

2.4. Corticotropin-releasing factor is the primary TSH releasing factor in tadpoles

Corticotropin-releasing factor and related peptides are potent stimulators of the thyroid axis in larval amphibians and other non-mammalian vertebrates via their direct stimulation of pituitary TSH secretion (Fig. 1B). The first evidence for this TSH releasing activity came from treating amphibian pituitary explants or primary pituitary cells with CRF peptides, and subsequent studies showed rapid elevations in whole-body TH content in tadpoles after injections of CRF peptides (Denver, 2013; Okada and Kikuyama, 2009; Watanabe et al., 2016). Injections of CRF-like peptides accelerated tadpole metamorphosis (Denver, 2013), metamorphosis of tiger salamander larvae (Boorse and Denver, 2002), and development of the direct developing frog *Eleutherodactylus coqui* (Kulkarni et al., 2010). Importantly, the majority of TSH releasing activity present in hypothalamic extracts from tadpoles and adult frogs (assayed using dispersed pituitary cells from adult frogs) was blocked by coincubation with the CRF receptor antagonist α-helical $CRF_{(9\text{-}41)}$ (Ito et al., 2004; Okada et al., 2009), thus supporting that TSH releasing activity in the amphibian hypothalamus is contributed primarily by CRF peptides. Hypothalamic CRF mRNA and peptide content increased during spontaneous metamorphosis in parallel with the rise in TH and CS production (Bender et al., 2018; Denver, 2009b, 2017).

The CRF_1 mRNA in tadpole pituitary was expressed during pre-metamorphosis, and increased during prometamorphosis, reaching a plateau at metamorphic climax (Manzon and Denver, 2004). This is consistent with tadpoles being able to mount a neuroendocrine stress response (elevation in CORT production) throughout the entire larval period (Glennemeier and Denver, 2002a). By contrast, the mRNA for CRF_2, which may be the primary receptor responsible for CRF stimulation of TSH secretion, was low or nondetectable during pre-metamorphosis and early prometamorphosis, but showed a large increase during late prometamorphosis and metamorphic climax (Manzon and Denver, 2004). This increase paralleled the increase in the sensitivity of the pituitary gland to secrete TSH after stimulation by CRF peptides (Kaneko et al., 2005) and the large increase in TH and CS production at climax (Denver, 2017). Taken together, the findings support that CRF and related peptides control tadpole metamorphosis by inducing pituitary TSH secretion via CRF_2, and the competence of the thyrotropes to respond to CRF peptides may depend on upregulation of CRF_2 at metamorphic climax.

2.5. Roles for CRF peptides in peripheral tissues

Components of the CRF signaling system are expressed throughout the body (Boorse and Denver, 2006; Slominski et al., 2013). Corticotropin-releasing factor peptides have cytoprotective function, to protect neural, cardiac and other cells from apoptosis (Alderman et al., 2018; Brar et al., 2002; Davidson et al., 2009; Jonassen et al., 2012; Linden et al., 2005; Martin et al., 2005; Radulovic et al., 2003; Szabadfi et al., 2009; Tao et al., 2006; Williams and Bernier, 2020). In tadpoles, CRF is expressed by tail muscle cells and acts in an autocrine manner as a cytoprotective factor for tail muscle cell survival during metamorphosis (Boorse et al., 2005). Corticotropin-releasing factor, acting via CRF_1, slowed spontaneous tail regression in tadpole tail explant cultures, which was paralleled by a reduction in caspase 3/7 activity. These findings in the tadpole are supported by findings in zebrafish showing that CRF repressed caspase 3 via a CRF_1-dependent pathway, and protected embryos from heat stress-induced apoptosis (Alderman et al., 2018). These peptides can also promote cell proliferation (Ikeda et al., 2002; Jessop et al., 1997; Mitsuma et al., 2001); CRF increased DNA synthesis in tadpole tail myoblast tissue culture cells (Boorse et al., 2005).

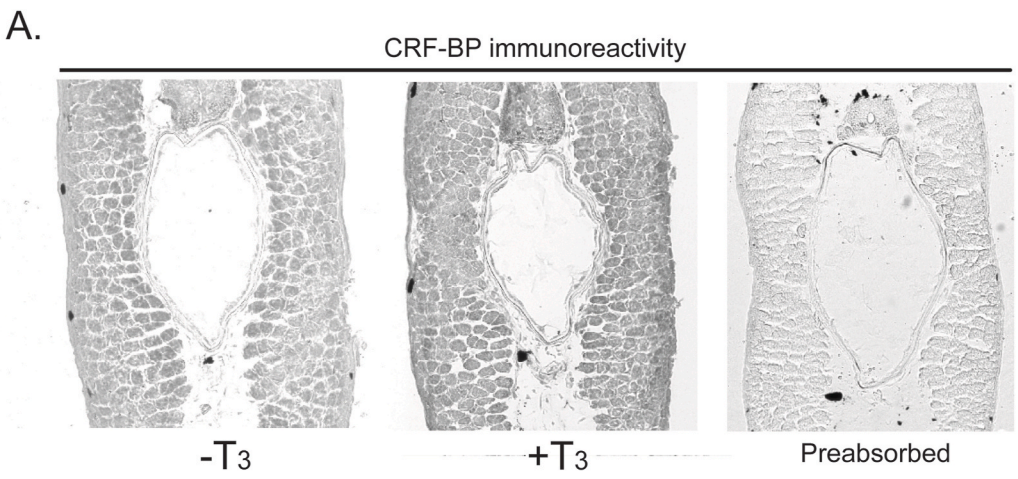
The CRF-BP modulates the cytoprotective actions of CRF in tadpole tail. It is expressed in tadpole tail, its mRNA level increased during spontaneous metamorphosis, and the mRNA and protein were strongly induced by treatment with TH (Boorse et al., 2006; Brown et al., 1996; Valverde et al., 2001) (Fig. 2A). The CRF-BP bound and neutralized the actions of CRF on cAMP production and [H^3]-thymidine incorporation in tail myoblast cells (Boorse et al., 2006). Forced expression of CRF-BP in tadpole tail *in vivo* using electroporation-mediated gene transfer accelerated the loss of tail muscle cells during spontaneous metamorphosis (Fig. 2B) (Boorse et al., 2006). Taken together, the data support that modulation of CRF bioavailability by CRF-BP promotes tail regression at metamorphic climax by neutralizing the cytoprotective actions of CRF (Boorse et al., 2006).

The tadpole tail is an essential locomotory organ required for feeding and escape from predators, and the developmental significance of CRF's cytoprotective role may be to maintain its viability until the animal is ready to transition to the adult stage. Environmental insults such as thermal or osmotic stress, hypoxia, hypercapnia, and tissue damage caused by predator attack could negatively impact tadpole tail cell survival and organ viability. When tadpole tail explants were exposed to hypoxia there was an increase in CRF and urocortin 1 mRNAs, but a strong decrease in CRF-BP mRNA (Boorse et al., 2006). The upregulation by environmental stressors of CRF and urocortin 1, and the coordinate downregulation of CRF-BP suggests that the production and bioavailability of CRF peptides, with their cytoprotective actions, can be modulated by direct environmental effects on the tadpole tail.

2.6. Corticosteroids and their nuclear receptors

The corticosteroids, produced by adrenal cortical (or interrenal) cells, are the primary effectors of the HPA/HPI axis. These hormones fall into two groups, the glucocorticoids and the mineralocorticoids, owing to their differential regulation, and often distinct physiological functions. The major physiological actions of CSs are mediated through binding to intracellular receptors that are members of the steroid receptor superfamily (Mangelsdorf et al., 1995). In vertebrates there are two types of CS receptors, originally identified in mammals based on their differential binding affinities for radiolabeled CS. These include the high affinity type I receptor (also called the mineralocorticoid receptor) and the lower affinity type II receptor (also called the glucocorticoid receptor; GR). Homologous receptor genes have been isolated in diverse vertebrate species including *Xenopus* frogs (Csikos et al., 1995; Gao et al., 1994a,b). Corticosteroids have diverse actions in animal development, physiology and behavior. They influence development of the brain, lungs and other organ systems, mobilize stored energy and stimulate feeding to replenish depleted energy stores following a stress response. In adults they have important effects on the brain to modulate learning and memory consolidation. Corticosteroids exert negative feedback at the level of the brain and pituitary gland to reduce the activity of the HPA/HPI axis, thus returning the system to baseline following exposure to a stressor (Yao and Denver, 2007; Yao et al., 2008b).

Amphibian interrenal glands produce CORT (and to a lesser extent cortisol and aldosterone) (Krug et al., 1983), and during tadpole metamorphosis CORT production increases in parallel with TH (Denver, 2017; Kikuyama et al., 2019). Glucocorticoids synergize with TH to promote tissue transformations during metamorphosis (discussed below) (Denver, 2017; Kulkarni and Buchholz, 2014). Recent work from Dan Buchholz's laboratory in which they inactivated the gene that codes for GR using CRISPR/Cas9 genome editing in *X. tropicalis* showed that GR is essential for survival through metamorphosis (Sterner et al., 2020). This same group inactivated the gene that codes for



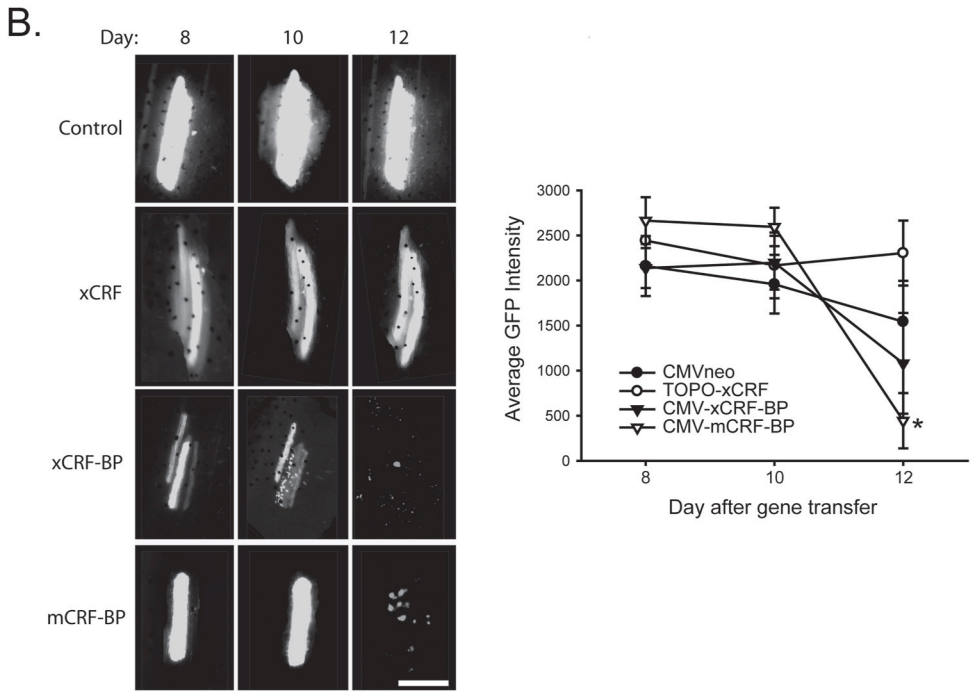


Fig. 2. The corticotropin-releasing factor (CRF) binding protein (CRF-BP) is expressed in tadpole tail where it modulates CRF bioavailability. Corticotropin-releasing factor is cytoprotective in the tadpole tail, helping to maintain tail viability for locomotion. At metamorphic climax the tail resorbs, and at this time the CRF-BP is upregulated under the control of thyroid hormone (TH). **A.** Immunoreactivity for CRF-BP can be induced preciously by treatment of premetamorphic tadpoles with TH. Nieuwkoop-Faber stage 50 tadpoles were treated with vehicle or 100 nM 3,5,3′-triiodothyronine (T_3) for 4 days by addition to their aquarium water. Shown are representative transverse cryosections (12 μm) of tadpole tail immunostained with an affinity-purified rabbit polyclonal antiserum (#3809; used at 1:30 dilution) generated against a synthetic peptide corresponding to amino acids 112–125 of *Xenopus* CRF-BP (FDGWIIKGEKFPSS) conjugated to keyhole limpet hemocyanin. Immune complexes were revealed using a goat anti-rabbit IgG conjugated to horse radish peroxidase using the Vectastain elite ABC kit and Vector VIP kit (both from Vector Laboratories, Inc., Burlingane, CA, USA). The right most panel shows a representative tail section from a TH-treated tadpole stained with anti-CRF-BP serum that had been preabsorbed with the synthetic peptide used as immunogen. The affinity purified antiserum was incubated with synthetic *Xenopus* CRF-BP peptide (100 μg/ml) in a 50 μl volume overnight at 4 °C before immunohistochemistry. All procedures involving animals were conducted in accordance with the guidelines of the University Committee on the Care and Use of Animals of the University of Michigan. **B.** Representative images of GFP expression in tadpole tail muscle cells (NF stage 58) *in vivo* after electroporation-mediated gene transfer. Muscle cells were co-electroporated with pEGFP-N3 and one of the following: TOPO-xCRF, CMV-xCRF-BP (*Xenopus* CRF-BP expression vector), CMV-mCRF-BP (mouse CRF-BP expression vector), or CMVneo (empty vector). Tadpoles were then reared in aquaria and GFP fluorescence imaged every two days thereafter. The graph to the right shows the quantification of the average GFP intensity in electroporated tail muscle cells over the final 4 days of the experiment. Shown are the means ± SEM (n = 6–8/treatment). * Significant difference from CMV-Neo transfected cells (Scheffe's post hoc test, $P < 0.05$) (reprinted from Boorse et al., 2006).

proopiomelanocortin, the precursor for ACTH and other bioactive peptides, and found that the tadpoles did not survive metamorphosis, but this defect could be rescued by treatment with CORT (Shewade et al., 2020).

3. Stress, hormones and developmental plasticity

Developmental plasticity can generate adaptive morphological, physiological, or behavioral traits in amphibians that promote survival

during larval life and after metamorphosis. Hormones, through their regulation of gene expression, play essential roles in the coordination of environmental and genetic information in the expression of suites of phenotypic traits, and therefore play key roles in determining fitness (Gilbert and Epel, 2008; Lema, 2014, 2020; West-Eberhard, 2005b). The amphibian neuroendocrine stress axis mediates physiological and behavioral responses to environmental change (Denver, 2009b, c). Here I review three aspects of developmental plasticity in amphibians and the roles that stress hormones play in these processes.

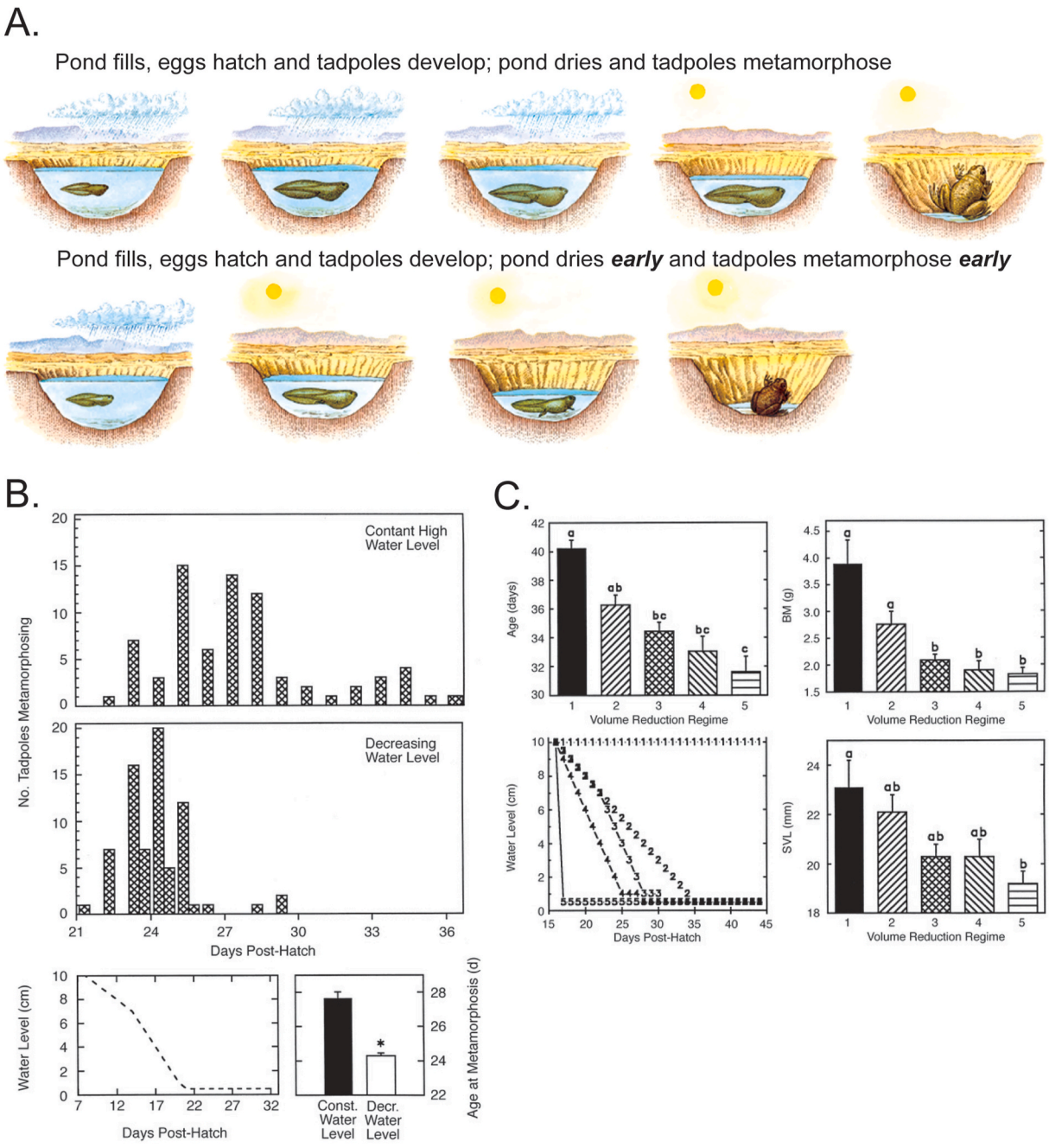


Fig. 3. Tadpoles accelerate metamorphosis when their pond dries. A. Graphic showing the acceleration of metamorphosis in response to pond drying in amphibian species that breed in arid environments, such as the Western spadefoot toad, *Spea hammondii*. Tadpoles that accelerate development in response to pond drying metamorphose earlier and at a smaller body size. This leads to tradeoffs between survival in the larval habitat and reduced post-metamorphic performance owing to smaller body size (graphic by Roberto Osti reprinted with permission; the graphic is based on original artwork by Leif Saul). **B.** Acceleration of metamorphosis of *S. hammondii* tadpoles caused by water volume reduction in the laboratory. The water level was maintained at either a constant high level (10 L) or decreasing (from 10 to 0.5 L; see bottom left panel). The top two panels show the frequency distributions of the two treatments for the numbers of animals metamorphosing by time since hatching. The bottom right panel shows the mean age at metamorphosis for the two treatments (mean ± SEM; n = 4 tanks/treatment; the asterisk indicates significant difference at $p < 0.001$; Student's unpaired t-test). **C.** The developmental response of *S. hammondii* tadpoles to a decreasing water level varied continuously in relation to the rate of change in the water level. Prometamorphic tadpoles (10 animals/tank) were exposed to different volume reduction regimes as shown in the bottom left panel (regimes 1–5). The bar graphs show mean age, body mass (BM) and snout-vent length (SVL) at metamorphosis (n = 3 tanks/treatment); error bars represent SEM (reprinted from Denver et al., 1998).

3.1. The timing of tadpole metamorphosis

The duration of the larval period is a central amphibian life history trait, and it varies considerably among and within species (Denver, 1997c). Each species has a minimum size for when metamorphosis can initiate, and the time that it takes to reach this minimum size is determined in large part by growth opportunity in the larval habitat (Werner, 1986; Wilbur and Collins, 1973). Variation in the larval habitat determines tradeoffs between growth opportunity and mortality risk, which influences the length of time that the animal spends as a tadpole. Species that breed in permanent, predictable habitats typically have relatively long larval periods (e.g. up to three years or even longer), while species that breed in unpredictable, often ephemeral ponds typically have short larval periods (as short as 10 days from hatching in some desert-adapted spadefoot toads) (Denver, 1997c; Denver et al., 2002).

The environmental conditions that tadpoles experience can have profound effects on their behavior, body size and morphology, and the timing of metamorphosis, all of which can strongly influence their survival (Denver et al., 1998; Goater, 1994; Newman, 1992; Relyea, 2007; Tarvin et al., 2015; Werner, 1986; Wilbur and Collins, 1973). Amphibians breed and lay their eggs in water, and so water is arguably the most important environmental variable for an amphibian tadpole. Drying of the natal pond accelerates metamorphosis in many amphibian species (Fig. 3) (Denver, 1997c; Newman, 1992). This acceleration is adaptive for amphibians that live in arid environments since it can increase the probability of survival to metamorphosis, and subsequently reproduction (Denver et al., 1998; Newman, 1992). However, there may be future fitness costs to accelerated metamorphosis owing to the tradeoff with smaller body size and fat reserves at transformation (Blouin and Brown, 2000; Burraco et al., 2017a), which can lead to reduced adult performance (Denver and Middlemis-Maher, 2010). This body size disadvantage at the time of metamorphosis may be retained through the age at first reproduction resulting in compromised reproductive fitness (Chelgren et al., 2006; Denver and Middlemis-Maher, 2010; Smith, 1987).

3.1.1. CRF peptides

While the timing of metamorphosis is determined by environmental factors such as pond duration, resource availability, predation pressure, etc., the initiation and progress of metamorphosis is regulated by the production, metabolism and actions of hormones. The competence of a tadpole to respond to environmental signals to initiate or accelerate metamorphosis depends on the maturation and activity of endocrine glands that produce the hormones that control metamorphosis. The activity of the tadpole HPT axis determines when tadpoles initiate metamorphosis, and also the rate at which metamorphosis progresses. Because the HPT and HPI axes in tadpoles communicate and cross-regulate at different levels, central nervous system stress pathways play a pivotal role in transducing environmental information and regulating metamorphic timing. With deteriorating environmental conditions (e.g., decreased resource availability, increased predation risk, pond drying) tadpole growth is reduced. If these conditions are experienced during early tadpole development before the animals initiate metamorphosis their development rate is slowed. By contrast, when tadpoles achieve the minimum body size for transformation and become competent to upregulate TH production and respond to the TH signal, they can respond to adverse environmental conditions by accelerating metamorphosis (Denver, 2009a).

Owing to their neurosecretion being induced by environmental stressors, and their stimulatory actions on pituitary TSH and ACTH secretion, CRF peptides are ideally positioned to play a central role in mediating a tadpole's developmental response to a deteriorating larval habitat (Boorse and Denver, 2004; Denver, 1997a, 1998; Denver et al., 1998). Tadpoles of the Western spadefoot toad (*Spea hammondii*) and other spadefoot toad species exposed to simulated pond drying increased whole-body TH and CORT content and accelerated metamorphosis (Boorse and Denver, 2004; Burraco et al., 2017b; Denver, 1997a, 1998; Denver et al., 1998; Gomez-Mestre et al., 2013). Several lines of evidence support that CRF peptides control the acceleration of metamorphosis caused by pond drying. For example, injections of CRF peptides accelerated metamorphosis in several amphibian species, including the Western spadefoot toad (Fig. 4A), and they increased whole-body TH and CORT content (Fig. 4B), consistent with CRF's known activity on tadpole thyrotropes and corticotropes (Denver, 1997a; Watanabe et al., 2016). Furthermore, hypothalamic CRF content increased after exposure to low water in spadefoot toad tadpoles that accelerated metamorphosis in response to simulated pond drying (Denver, 1997a). Importantly, blockade of CRF action or bioavailability by injection of the CRF receptor antagonist alpha-helical $CRF_{(9\text{-}41)}$ or by passive immunization with anti-CRF serum, respectively, blocked the acceleration of metamorphosis caused by pond drying (Fig. 4C) (Denver, 1997a).

In addition to pond drying, tadpoles experience variation in conspecific density, food resources and predation risk, which can affect the timing of metamorphosis. Evidence supports that this environmental information is transduced by the neuroendocrine stress axis. For example, food restriction or high conspecific density increased whole-body CORT content of premetamorphic tadpoles, slowed growth and development, and reduced cell proliferation and increased apoptosis in neuroendocrine centers of the brain (Distler et al., 2016; Glennemeier and Denver, 2002b; Hayes, 1997). The reduction in premetamorphic tadpole growth rate caused by crowding was reversed by treatment with the CORT synthesis inhibitor metyrapone (Glennemeier and Denver, 2002b). By contrast, prometamorphic tadpoles accelerated development in response to food restriction or crowding, owing to the maturation at this developmental stage of their hypophysiotropic brain centers controlling TSH secretion (Denver, 2017). Predation, temperature, photoperiod, or other environmental factors may also operate via similar neuroendocrine pathways to modulate larval growth and development, and the timing of metamorphosis.

3.1.2. Hormone action at target tissues

In addition to hypothalamic and pituitary factors, the regulation of the timing and progression of metamorphosis depends on the bioavailability and actions of TH and CS at target tissues (Denver, 2017). The availability of biologically active TH is regulated within tissues by membrane and intracellular transporters, and monodeiodinase enzymes (Choi et al., 2015; Denver, 2013). A role for TH uptake into cells and/or TH metabolism in modulating larval period length is suggested by studies in closely related species of spadefoot toads that differ in the duration of their larval periods (Buchholz and Hayes, 2005). These species show strong differences in TH tissue content, and the sensitivity of their tissues to TH, which was positively correlated with larval period length (Buchholz and Hayes, 2002; Hollar et al., 2011; Kulkarni et al., 2017). The expression level of TRs influences cellular sensitivity to the TH signal (Hollar et al., 2011; Hu et al., 2016; Nakajima et al., 2012), and is negatively correlated with larval period length in different spadefoot toad species, with higher TR levels associated with shorter larval periods (Hollar et al., 2011).

3.1.3. Corticosteroid synergy

Exposure to stressors during larval life activates the HPI axis, and CSs have complex effects on tadpole growth and development (Denver, 2017). Premetamorphic tadpoles are typically voracious feeders focused on maximizing growth to reach the minimum body size to initiate metamorphosis. Stressors experienced during this developmental stage can have negative effects on growth and development. By contrast, environmental stress experienced during prometamorphosis elevates TH and CS production, and since tadpoles are now competent to upregulate the HPT axis they can accelerate metamorphosis. This acceleration may enhance fitness by allowing the animal to escape a deteriorating larval

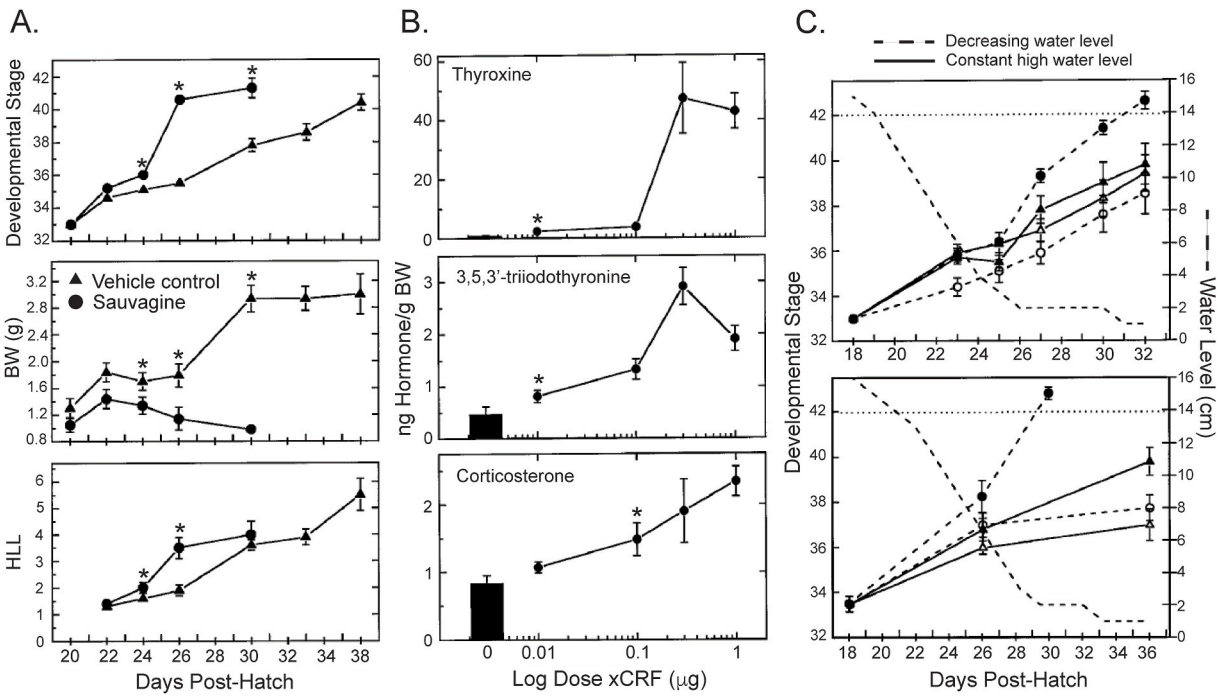


Fig. 4. Corticotropin releasing factor peptides accelerate tadpole metamorphosis. A. Acceleration of tadpole metamorphosis by the CRF peptide sauvagine (SV). Premetamorphic *Spea hammondii* tadpoles were injected intraperitoneally with vehicle or SV every other day for 10 days. Shown are changes in Gosner (Gosner, 1960) developmental stage (top), body weight (BW) and hind limb length (HLL). Each point is the mean + SEM (n = 10–12 animals/time point), and asterisks indicate statistically significant differences between vehicle and SV ($p < 0.05$). **B.** Injection of synthetic *Xenopus* CRF caused a rapid (by 6 h), dose-dependent increase in whole-body thyroxine, 3,5,3′-triiodothyronine and corticosterone content in *S. hammondii* tadpoles. Asterisks indicate the minimum effective dose; this dose and all doses higher were significantly different from the zero dose ($p < 0.05$; Student's unpaired t-test). **C.** Antagonism of endogenous CRF by injection of the CRF receptor antagonist a-helCRF$_{(9–41)}$ (top; open symbols = a-helCRF$_{(9–41)}$, closed symbols = vehicle) or rabbit antiserum to frog CRF (bottom; open symbols = anti-CRF, closed symbols = normal serum) blocked the developmental response of *S. hammondii* tadpoles to experimental pond drying. Dashed lines and circles indicate a decreasing water level, while solid lines and triangles indicate a constant high water level. Each point is the mean + SEM (n = 8 animals/treatment and time point). Metamorphic climax (Gosner stage 42) is indicated by the horizontal dotted line in the upper part of the graph (reprinted from Denver, 1997b).

habitat and transition to the next life history stage (Denver, 1997c, 2009a; Denver and Middlemis-Maher, 2010; Kulkarni and Buchholz, 2014).

A key mechanism discussed above that allows prometamorphic tadpoles to accelerate metamorphosis is the activation of the HPT and HPI axes by CRF peptides. Another important mechanism is the synergism between TH and CS at target tissues (Fig. 5) that promote transcription of genes that drive metamorphosis (Kulkarni and Buchholz, 2014; Sachs and Buchholz, 2019). For example, CS can induce expression of TRs to increase cellular sensitivity to the hormone signal (Bonett et al., 2010; Kikuyama et al., 1993; Niki et al., 1981; Suzuki and Kikuyama, 1983), especially TR beta which is necessary for proper completion of metamorphosis (Nakajima et al., 2019; Shibata et al., 2020a, 2020b). The actions of CSs were synergistic with low or subthreshold doses of TH, producing strong induction of TR expression (Bonett et al., 2010). Also, CORT can enhance TH bioactivity within tadpole cells by increasing 5′-deiodinase activity and *deiodinase* 2 mRNA (Bonett et al., 2010; Darras et al., 2002; Galton, 1990; Kuhn et al., 2005), and this action of CORT was synergistic with TH in tadpole tail (Bonett et al., 2010); see also studies in the axolotl (Darras et al., 2002; Kuhn et al., 2005). Similar hormone synergy has been reported in larval fish (Brown et al., 2014).

In addition to the TRs and monodeiodinases, synergistic regulation by TH and GC has been found for other genes expressed during metamorphosis (Bagamasbad et al., 2015; Kulkarni and Buchholz, 2012), and some TH and GC target genes are synergistically induced via a mechanism that is not dependent on an increase in TRs or monodeiodinases. That is, TH and CS together cause synergistic activation of transcription of genes that are directly regulated by liganded TR and GR/MR. For example, *krüppel-like factor 9*, a direct TH target gene, is induced by CORT (Bonett et al., 2009), and is synergistically induced in tadpole tissues by combined treatment with TH plus CORT (Bagamasbad et al., 2015). Similar synergistic regulation of *krüppel-like factor* 9 by TH and CSs was seen in mouse brain and neuronal cells, and the synergy was mediated by an ultraconserved superenhancer with binding sites for TR and GR/MR (Bagamasbad et al., 2015, 2019). There are other genes that are synergistically regulated by TH and CS, and their protein products may function to accelerate metamorphosis (Kulkarni and Buchholz, 2012, 2014). The common regulation of the HPT and HPI axes by CRF peptides, and the sensitization of target tissues to low concentrations of TH by CS provides a mechanism for tadpoles to modulate their rate of development in response to a changing environment.

3.2. *Behavioral and morphological plasticity*

Amphibian larvae have extraordinary capacity for behavioral and morphological plasticity (Chipman, 2002; Newman, 1989). For example, while most amphibian tadpoles are omnivores that feed on detritus on the pond floor, the larvae of some species of frogs and salamanders respond to the presence (and type) of prey by developing distinct carnivore morphologies that favors growth and development with the higher quality food source (Levis and Pfennig, 2019; Michimae et al., 2005; Michimae and Wakahara, 2001, 2002; Pfennig, 1990, 1992). The development of two or more distinct phenotypes from the same genotype, as in the case of the omnivore vs. carnivore morphology of some amphibian larvae, is often referred to as polyphenism.

Another important driver of polyphenism in tadpoles is the presence or absence of predators in the larval habitat. Predators strongly influence tadpole behavior and rates of growth and development, and can generate polyphenisms that promote survival in the larval habitat. In

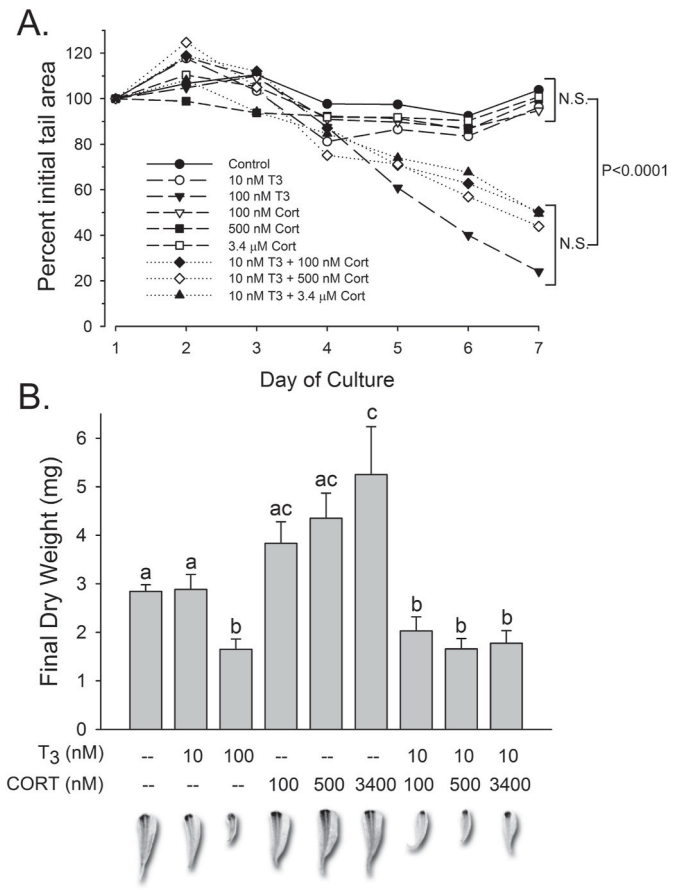


Fig. 5. Corticosterone synergizes with thyroid hormone to accelerate tadpole metamorphosis. Tail explants from premetamorphic *Xenopus laevis* tadpoles were cultured for one week in the presence of 3,5,3′-triiodothyronine (T_3) or corticosterone (CORT) or both as indicated. **A.** Changes in the percent initial tail area over the 7 day culture period are shown. Each point is the mean ± SEM (n = 7/treatment; the statistical analysis was done on the actual tail areas; one-way ANOVA). **B.** Changes in the final dry weight of tadpole tail explants. The bars represent the mean ± SEM (n = 7/treatment; means with the same letter are not significantly different; Fisher's LSD post hoc test, $p < 0.05$). Representative images of tails from each treatment group at the 7 day time point are shown below the graphs (reprinted from Bonett et al., 2010).

addition to visual cues, tadpoles can detect predators via chemical cues emitted by predators (kairomones) and by conspecifics (alarm pheromones) (Benard, 2004; Hettyey et al., 2012, 2015; Mitchell et al., 2017; Relyea, 2007). Tadpoles respond rapidly to predator presence by behavioral inhibition (freezing) which facilitates avoidance of detection by the predator (Fraker, 2008; Mitchell et al., 2017). Tadpole alarm pheromones (also referred to as predator chemical cues) are produced by skin cells and cause rapid freezing behavior (Fig. 6A) (Fraker et al., 2009). The alarm pheromone is released by mild damage to the tail skin (poking with a hypodermic needle), and it appears to be present within secretory vesicles since it is released upon homogenization of tadpole tissue in the presence of detergent (Triton X-100). Also, it can be released by exposure of living tadpoles to potassium chloride, which depolarizes cell membranes to cause release of secretory vesicles, supporting that the alarm pheromone is secreted via a stimulus-secretion coupled pathway (Fig. 6A). Analysis of its biochemical properties is consistent with it being a polypeptide(s) (although see Austin et al., 2018 for evidence for other, non-peptide components), and purification by reversed-phase high performance liquid chromatography showed that it is comprised of two biochemically distinct components that must be combined to elicit the behavioral response (Fraker et al., 2009).

Tadpole behavioral and morphological responses to predators are linked to hormones of the neuroendocrine stress axis (Harris and Carr, 2016; Middlemis-Maher et al., 2013). In mammals the HPA axis is rapidly activated in response to predator cues (Apfelbach et al., 2005; Figueiredo et al., 2003; Hegab and Wei, 2014; Roseboom et al., 2007). This appears to also be true in tadpoles where exposure to alarm pheromone caused an early HPI axis response (1–30 min) measured by increased whole-body CORT content (Bennett et al., 2016). However, unlike in mammals, tadpoles suppress their HPI axis 1–4 h after exposure to the alarm pheromone, as evidenced by a 70% decline in whole-body CORT content (Fig. 6B) (Fraker et al., 2009). This suppression is important for maintaining behavioral inhibition with continued predator presence, as shown by the resumption of activity in tadpoles exposed to alarm pheromone after treatment with CORT (to reverse the decline caused by the alarm pheromone; Fig. 6C); both exogenous and endogenous CORT stimulated tadpole locomotion and foraging (Crespi and Denver, 2004). The behavioral inhibition supported by suppression of the HPI axis likely plays a critical role in tadpole survival. Predator-naïve tadpoles treated with CORT (for 3 h) and exposed to lethal predators (i.e., uncaged dragonfly larvae) had lower survivorship compared with vehicle-treated controls (Middlemis-Maher et al., 2013).

Tadpoles can adjust their behavior over time in proportion to the level of risk in the environment (Fraker, 2009, 2010; Lucon-Xiccato et al., 2016). Also, predation risk that extends over days to weeks leads to the development of distinct anti-predator morphology which can have indirect effects on fitness by allowing tadpoles to avoid predators while continuing to forage and grow (Benard, 2004; Relyea, 2007). For example, chronic exposure to predators (nonlethal, caged) or to alarm pheromone caused changes in coloration of the tadpole tail (an aposematic response), increased tail height and decreased body size (Fig. 7A) (Benard, 2004; McCollum and Leimberger, 1997; Relyea, 2001b; Van Buskirk and Mccollum, 1999). The larger tail may serve to lure predator attacks away from the more vulnerable body (see tail damage from predator attack in Fig. 7A), and can improve escape from predators through enhanced burst locomotion (Fraker et al., 2020; Van Buskirk and McCollum, 2000). Chronic predator presence also results in complex effects on the timing of and size at metamorphosis (Benard, 2004; Fraker, 2008; Fraker et al., 2009; Relyea, 2007).

While the acute effect of the alarm pheromone is to first activate, then suppress the HPI axis, longer exposure (4 days or more) increased CORT production (Fig. 7B). Tadpoles exposed to alarm pheromone increased whole-body CORT content ~2 fold at 4 days, which remained elevated through 8 days of continuous exposure. In these experiments, tadpoles exposed to alarm pheromone for two weeks showed allometric changes in body morphology, developing larger tails and smaller bodies (Fig. 7C) (Middlemis-Maher et al., 2013) as seen previously with nonlethal exposure to predators (Benard, 2004; McCollum and Leimberger, 1997; Relyea, 2001b; Van Buskirk and Mccollum, 1999). Treatment with CORT by addition to the aquarium water over the same time period generated a similar anti-predator morphology, and importantly, the effect of the alarm pheromone on tadpole morphology was blocked by co-treatment with the CS synthesis inhibitor metyrapone (MTP) (Fig. 7C). Hossie and colleagues (Hossie et al., 2010) found similar effects of MTP in blocking predator-induced changes in tail morphology in tadpoles of the leopard frog. Similar effects of CORT on tadpole tail size have been observed in other species (Bonett et al., 2010; Glennemeier and Denver, 2002c; Middlemis-Maher et al., 2013). The anti-predator morphology has important fitness consequences, as evidenced by increased survivorship in lethal predator trials of tadpoles treated for 8 days with CORT (which induces the change in morphology) compared with tadpoles treated with vehicle or MTP (Middlemis-Maher et al., 2013). Taken together, the findings show that changes in tadpole tail and body morphology caused by predator presence, which enhances tadpole survival, are mediated by CSs.

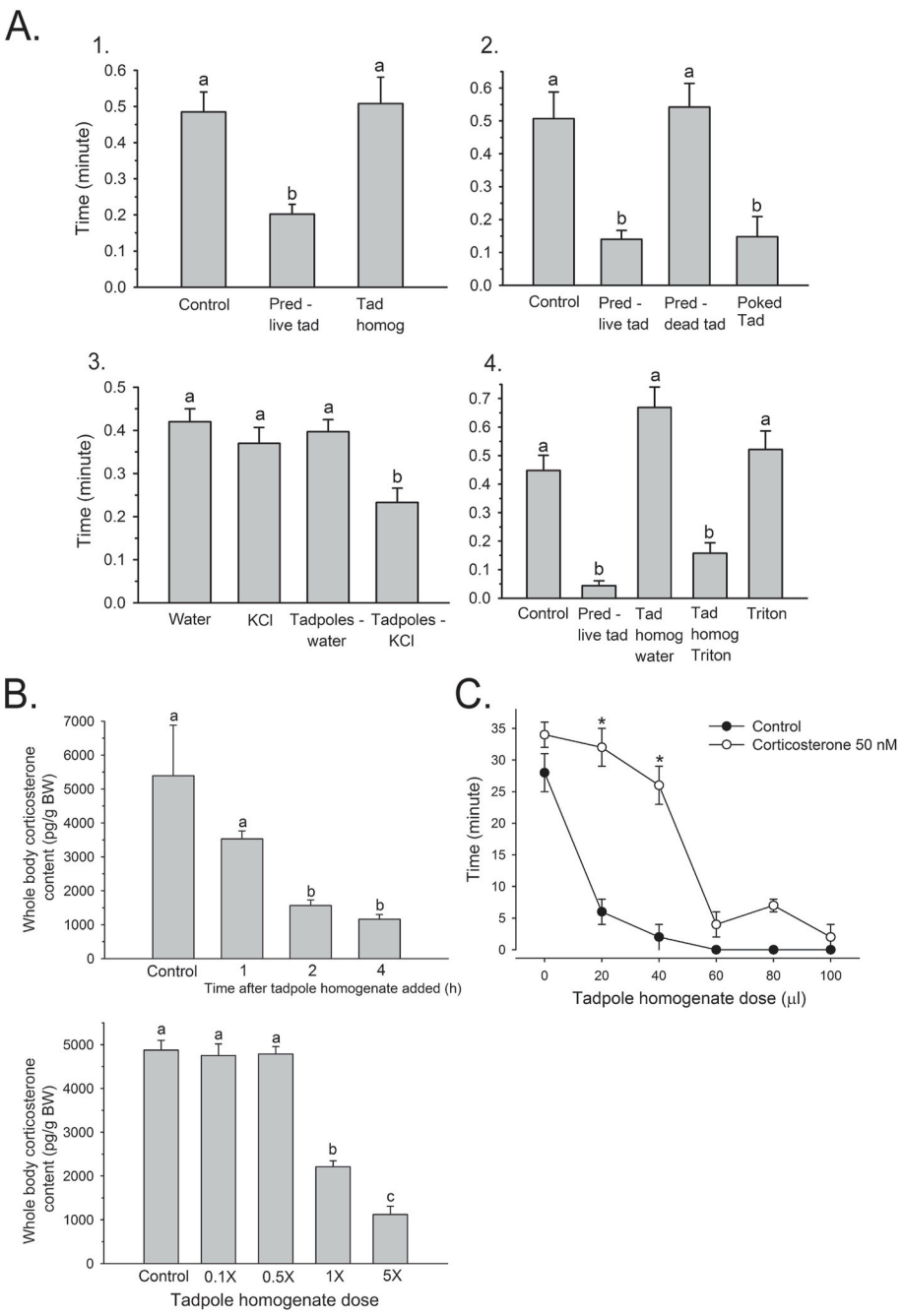


Fig. 6. Ranid tadpoles release an alarm pheromone from their skin when attacked by a predator that causes rapid freezing behavior and suppression of the HPI axis in conspecifics. A. An alarm pheromone is produced by ranid tadpoles and is released via a stimulus-secretion coupled pathway. The graphs show the mean time spent swimming of tadpoles exposed to the different treatments. (1) Tadpoles reduced activity when exposed to predator-conditioned water (dragon fly larvae fed live tadpoles) but not by euthanized tadpoles homogenized in water. (2) Tadpoles reduced activity when exposed to water conditioned by tadpoles poked with a hypodermic needle, but not by dragon fly larvae fed dead tadpoles. (3) Tadpoles reduced activity when exposed to water conditioned by tadpoles that had been immersed in 5 mM potassium chloride (KCl), but not by tadpoles immersed in water alone or the KCl alone. (4) Tadpoles reduced activity when exposed to a homogenate made with euthanized tadpoles in 1% Triton X-100, but not tadpoles homogenized in water alone. Pred — predator; Tad — tadpole; homog — tadpole homogenate; Triton — Triton X-100. The bars show the mean ± SEM; means with the same letter within an experiment are not significantly different ($p < 0.05$). **B.** Exposure of tadpoles to alarm pheromone reduced whole-body corticosterone (CORT) content in a time and dose-dependent manner. Tadpoles were exposed to the tadpole Triton X-100 homogenate and then sacrificed at different times for analysis of whole-body CORT content (top graph). The alarm pheromone caused a dose-dependent suppression of tadpole whole-body CORT content (bottom graph). Tadpoles were exposed to the homogenate for 4 h before sacrifice and analysis of whole-body CORT content. **C.** Reversing the decline in endogenous CORT caused by exposure to the alarm pheromone through treatment with CORT partially blocked the anti-predator behavior. Corticosterone was added to the aquarium water to a final concentration of 50 nM. Controls received an equal volume of ethanol vehicle (final concentration 0.001%). Triton X-100 tadpole homogenate was added to the tanks in 20 µl aliquots at 15 min intervals. Shown is the mean time tadpoles spent swimming±SEM. Asterisks indicate significant differences between the CORT treated and control groups at the indicated doses ($p < 0.05$) (reprinted from Fraker et al., 2009).

3.3. Developmental programming

It is now established in species as evolutionarily distant as arthropods and humans that the environment experienced during early life stages can influence traits expressed in the juvenile or adult stage (Barker, 1997; Gilbert, 2016; Sultan, 2017, 2019). The effect on fitness depends on the context and informational content of the early experiences; some carry-over effects may be beneficial when the early-life experience prepares the organism for conditions encountered later in life (i.e., see the Thrifty Phenotype hypothesis; (Hales and Barker, 1992; Prentice, 2005). On the other hand, exposure to stressors early in life can lead to higher probabilities of reproductive dysfunction and adult-onset disease (Barker, 1997; Chen and Baram, 2016; Choe et al., 2019; Fogelman and Canli, 2019; Lux, 2018; Malik and Spencer, 2019; Murphy et al., 2017; Ridout et al., 2018; Wakeford et al., 2018; Walters and Kosten, 2019).

Phenotypic carry-over occurs between different stages of the amphibian life cycle and may have wide-ranging effects on individual fitness (Altwegg and Reyer, 2003; Alvarez and Nicieza, 2002; Goater, 1994; Scott, 1994; Van Buskirk and Saxer, 2001). One way that the developmental environment can affect later life performance is by altering metamorphic timing, which affects size at transformation (Blouin and Brown, 2000). Smaller juvenile frogs have lower rates of dispersal from their natal pond, and also survival to first reproduction compared with larger animals (Chelgren et al., 2006; Smith, 1987). Larger females reach reproductive maturity earlier, produce larger eggs and larger clutch sizes (Girish and Saidapur, 2000; Prado and Haddad, 2005; Scott, 1994). Earlier reproductive maturity increases the proportion of individuals that survive to reproduce, and thus can substantially increase the population growth rate (Birch, 1948; Cole, 1954) as well as individual fitness (greater lifetime fecundity) (McGraw and Caswell, 1996). Tadpoles exposed to pond drying accelerated metamorphosis and

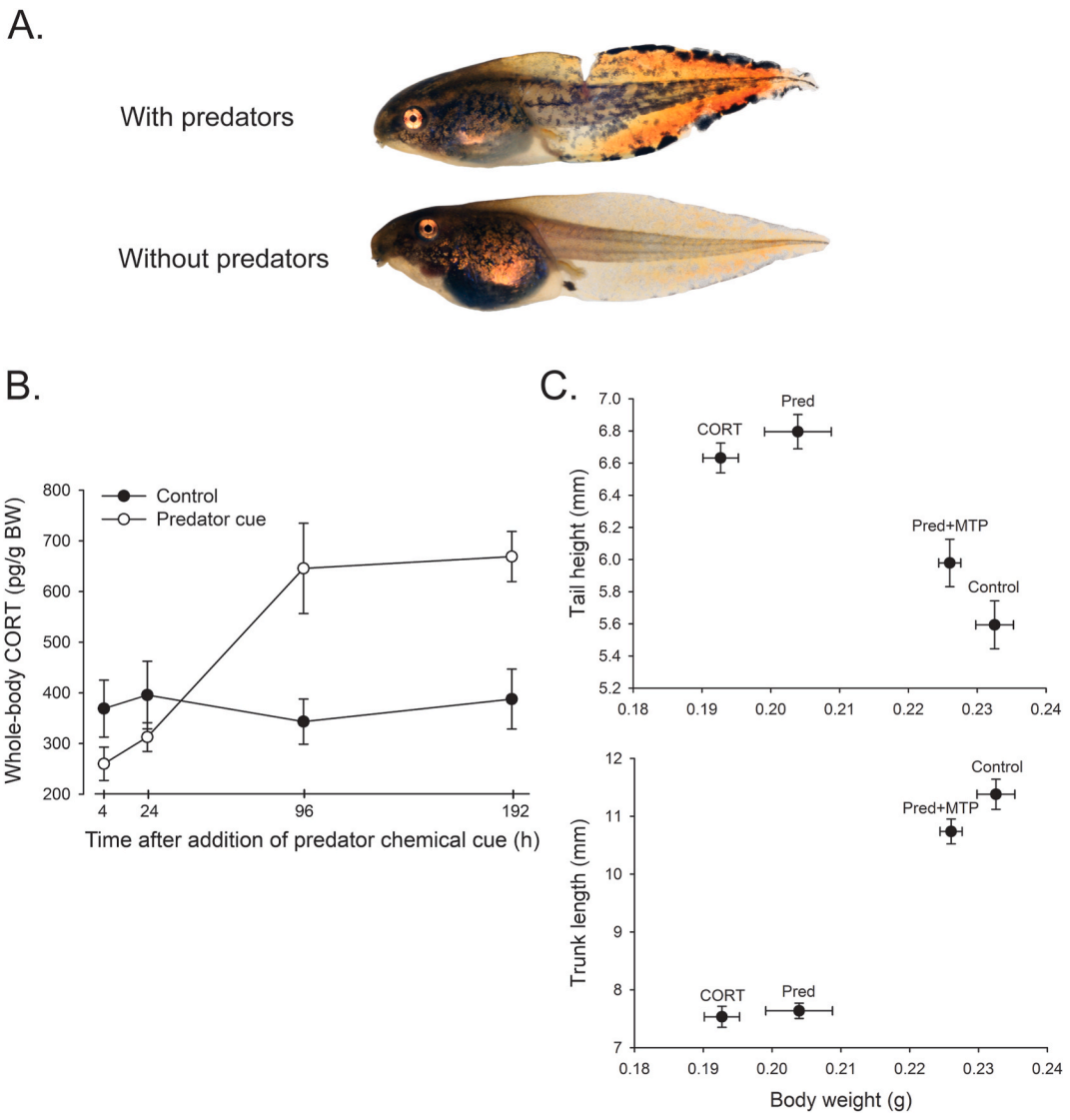


Fig. 7. Exposure to predators induced anti-predator morphology in wood frog tadpoles. A. Gray treefrog tadpoles exposed to predators developed tail coloration that discourages predation (an aposematic response), and changes in body morphology that enhances predator avoidance (photo by Michael Benard). **B.** Exposure of tadpoles to the non-lethal presence of predators caused a biphasic response in whole-body CORT content. Tadpoles were exposed to caged aeshnid predators fed conspecific tadpoles in mesocosms and sampled at the indicated times for measurement of whole-body CORT content. The tadpoles initially reduced whole-body CORT content at 4 h after exposure, but then elevated CORT content by 4 days after exposure (time × treatment interaction: $p < 0.001$; one-way ANOVA). Each point represents the mean ± SEM (n = 10 animals/treatment and time point). **C.** Exposure of tadpoles to alarm pheromone or CORT generated similar anti-predator morphology, and the effect of the alarm pheromone was blocked by co-treatment with the corticosteroid synthesis inhibitor metyrapone (MTP). Tadpoles were treated with alarm pheromone (Pred), CORT (125 nM) or Pred plus MTP (110 mM). Tail height (top graph) and trunk length (bottom graph) were corrected for body weight. Both measures were significantly affected by the treatments ($p < 0.05$). Tadpoles treated with alarm pheromone or CORT both developed deeper tails and shorter trunks compared with controls, and were not significantly different from each other in either measure. Tadpoles treated with alarm pheromone plus MTP had shallower tails and longer trunks than tadpoles treated with alarm pheromone alone. Each point is the mean ± SEM (n = 16/treatment) (reprinted from Middlemis-Maher et al., 2013).

metamorphosed at a smaller body size, which caused performance deficiencies in locomotion and immune function of juvenile frogs (Brannelly et al., 2019; Sinsch et al., 2020). On the other hand, frogs from tadpoles reared in the presence of predators had longer limbs, which provide for better jumping ability to escape predators (Emerson, 1978; Nicieza et al., 2006; Relyea, 2001a; Van Buskirk and Saxer, 2001).

Elevations in plasma [GC] during early development play a key role in programming the phenotype expressed in the juvenile/adult stage (Alyamani and Murgatroyd, 2018; Buschdorf and Meaney, 2016; Denver, 2009b; Fogelman and Canli, 2019; Hu et al., 2008; Liu and Nusslock, 2018; van Bodegom et al., 2017). In mammals, early life exposure to stressors that elevated plasma [GC] caused changes in the physiology and behavior of the juvenile/adult (Alyamani and Murgatroyd, 2018; Anacker et al., 2014; Buschdorf and Meaney, 2016; Fogelman and Canli, 2019; O'Donnell and Meaney, 2020; van Bodegom et al., 2017). A consistent finding is that prenatal stress leads to elevated basal plasma [GC] in the juvenile/adult (Alyamani and Murgatroyd, 2018; Buschdorf and Meaney, 2016; Fogelman and Canli, 2019; Liu and Nusslock, 2018; Meaney et al., 2007; van Bodegom et al., 2017). Amphibians appear to respond similarly to mammals; treatment of prometamorphic tadpoles with CORT (100 nM) added to their aquarium water for 5 or 10 days resulted in increased basal plasma [CORT] in juvenile frogs analyzed two months after metamorphosis (Hu et al., 2008).

The elevated plasma [GC] in juvenile/adult mammals caused by early life stress may result from impaired negative feedback caused by a reduction in GR expression in the brain (Alyamani and Murgatroyd,

2018; Buschdorf and Meaney, 2016; Fogelman and Canli, 2019; Liu and Nusslock, 2018; van Bodegom et al., 2017). Similarly, juvenile frogs that had received CORT treatment as tadpoles (100 nM in their aquarium water for 5 or 10 days) had less than half the amount of GR immunoreactivity (ir) in the anterior preoptic area (POA – location of hypophysiotropic CRF neurons in frogs, and homolog of the mammalian paraventricular nucleus - PVN) and the anterior pituitary gland analyzed by immunohistochemistry (Hu et al., 2008). The mean GR-ir was also reduced in limbic structures like the medial amygdala, bed nucleus of the stria terminalis, and the medial pallium (homologous to the

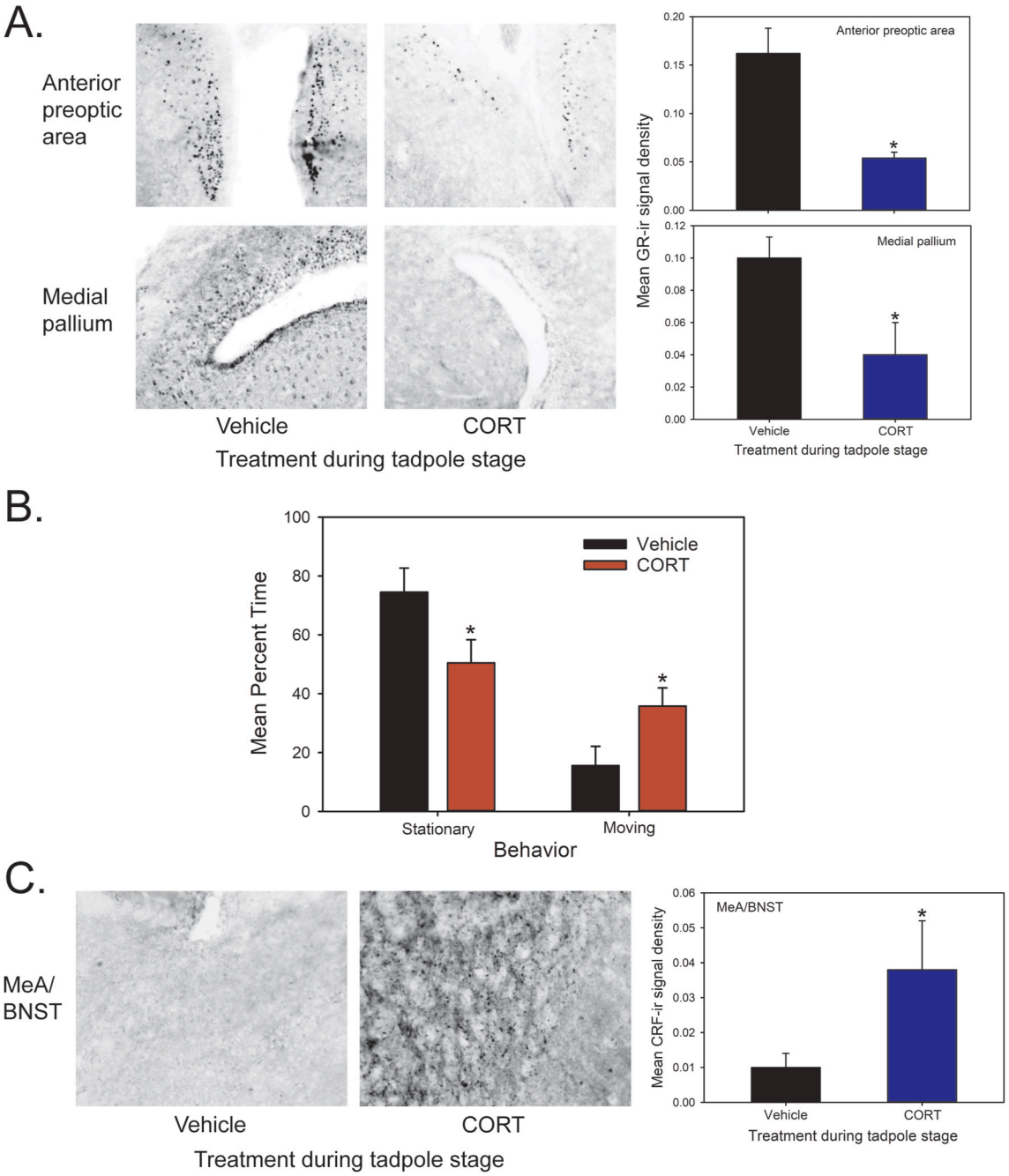


Fig. 8. Treatment of premetamorphic *Xenopus laevis* tadpoles with CORT to mimic a stress response decreased brain glucocorticoid receptor (GR), increased anxiogenic behavior and increased brain corticotropin-releasing factor (CRF) in juvenile frogs. Prometamorphic tadpoles (NF stage 56–57) were treated with vehicle (0.001% ethanol) or corticosterone (CORT; 100 nM) for 5 days by addition to their aquarium water as described (Hu et al., 2008). All analyses were done two months after metamorphosis. **A.** Treatment of prometamorphic tadpoles with CORT decreased GR-ir in the brains of juvenile frogs, (GR-ir analyzed on cryosections of frog brain as described by Yao et al., 2008a; n = 5/treatment). Shown are the anterior preoptic area (POa) and medial pallium (homolog of mammalian hippocampus). Similar changes in GR-ir were seen in the amygdala and bed nucleus of the stria terminals (BNST; not shown). **B.** Treatment of tadpoles with CORT increased anxiogenic behavior of juvenile frogs. Frogs were placed individually into tanks (n = 7/treatment), the baseline behavior was monitored for one hr (which did not differ between treatments – data not shown), then individual frogs were subjected to a single negative stimulus (tapping on the tank with a pen every two seconds for one minute with enough force to startle the frog). Frog behavior was recorded for one hr using a closed-circuit camera, and activity level was scored (time spent stationary or moving). **C.** Treatment of prometamorphic tadpoles with CORT caused a large increase in CRF-ir in the region of the medial amygdala (MeA) and BNST of the brains of juvenile frogs. The CRF-ir was analyzed on cryosections of frog brain as described (Yao et al., 2004; n = 5/treatment). * $p < 0.05$ Student's unpaired t-test.

mammalian hippocampus) (Fig. 8A). In mammals, these structures are involved in negative feedback regulation of PVN CRF neurons; similar regulation occurs in frogs (Yao and Denver, 2007; Yao et al., 2008a, 2008b). The activity of the HPA/HPI axis is regulated by GC negative feedback acting within the brain and pituitary, predominantly via the GR. The increase in basal plasma [CORT] and decrease in GR-ir in frogs that had been exposed to CORT as tadpoles (Hu et al., 2008) is consistent with reduced negative feedback within the HPI axis.

In mammals, exposure to early life stress can cause a diversity of psychopathologies (Chen and Baram, 2016; Fogelman and Canli, 2019; Lux, 2018; O'Donnell and Meaney, 2020; Vaiserman, 2015; Walters and Kosten, 2019). Neonatal stress in mammals causes stable, long term alterations in the morphology of CRF neurons in brain areas involved with neuroendocrine and behavioral responses to stress (i.e., PVN, amygdala, bed nucleus of the stria terminalis - BNST, hippocampus, locus coeruleus) (Buschdorf and Meaney, 2016; Meaney, 2001). Comparative studies support that the functions of limbic structures in the stress response are conserved in tetrapods (Carr, 2015; Daviu et al., 2019; Yao and Denver, 2007; Yao et al., 2004, 2008a, 2008b). The amygdala and BNST play central roles in fear and anxiety-related behaviors (Herman et al., 2005; Morgane et al., 2005; Schafe et al., 2005; Schulkin et al., 2005), and also influence neuroendocrine and autonomic functions (Herman et al., 2005; Morgane et al., 2005). In rodents and frogs, CRF neurons in the amygdala and BNST are activated in response to fear/anxiety-provoking stressors (Becker et al., 2007; Bruijnzeel et al., 2001; Casada and Dafny, 1991; Daviu et al., 2019; Gray, 1993; Kovacs, 2013; Makino et al., 1999; Merali et al., 1998; Rotllant et al., 2007). We found that treating prometamorphic tadpoles with CORT (100 nM for 5 days) resulted in elevated anxiogenic behavior (Fig. 8B) and a dramatic increase in CRF immunoreactivity in the brain of juvenile frogs measured two months after metamorphosis (Fig. 8C).

Taken together, the findings show that early-life exposure to GCs cause long-term changes in the activity of the HPI axis and increased anxiogenic behavior in frogs as in mammals. These changes produce physiological and behavioral modifications that may have important fitness consequences.

4. Summary and perspectives

The free-swimming larvae of species with complex life cycles are exposed to diverse biotic and abiotic factors in their habitats, which can have profound effects on phenotypic expression, both during larval growth and development, and later in the juvenile/adult. Stress hormones play pivotal roles in mediating environmental effects on development in vertebrates. In amphibian tadpoles, CRF acts as a central integrator of environmental stress to induce thyroid and interrenal hormone secretion, thereby modulating the timing of metamorphosis. Corticotropin releasing factor is a phylogenetically ancient modulator of development in vertebrates (Watanabe et al., 2016). Central nervous CRF neurons are sensitive to external and internal environmental factors, and allow animals to assess the quality of their developmental habitat to mount an appropriate developmental/physiological response (Denver, 2009b). In this regard, in mammals, maternal malnutrition or exposure to stressors cause intrauterine growth retardation and pre-term birth (Bloomfield et al., 2003; Challis et al., 2001; Weinstock et al., 1992, 1998). Corticotropin releasing factor of fetal and/or placental origin controls the timing of parturition, which is accelerated under conditions of fetal stress (Challis et al., 2005; Hillhouse and Grammatopoulos, 2002; Howland et al., 2017; McLean and Smith, 2001; Smith et al., 2002). Thus, the neuroendocrine stress axis is strongly influenced by environmental input, and is therefore a central, proximate mechanism for developmental plasticity. In tadpoles, the actions of TH at target tissues are enhanced by GC, acting to increase expression of TRs and monodeiodinases, and transactivate other genes important for morphogenesis (Bonett et al., 2010; Denver, 2009b). Whether similar mechanisms occur in mammals requires further investigation.

The effects of environmental stress on tadpole growth and development parallel those of intrauterine stress on fetal growth and development in mammals. In humans, intrauterine growth retardation and preterm birth are associated with elevated plasma [GC] in both mother and fetus, which permanently alters the functioning of the stress axis and the expression of behaviors throughout the life of the animal (Alyamani and Murgatroyd, 2018; Buschdorf and Meaney, 2016; Fogelman and Canli, 2019; Liu and Nusslock, 2018; Vaiserman, 2015; van Bodegom et al., 2017). Maternal malnutrition or exposure to stressors that cause intrauterine growth retardation are associated with reproductive dysfunction and increased susceptibility to disease later in life (Barker, 1997; Chen and Baram, 2016; Choe et al., 2019; Fogelman and Canli, 2019; Murphy et al., 2017; Ridout et al., 2018; Wakeford et al., 2018; Walters and Kosten, 2019; Zulma et al., 2017). The actions of GCs in programming long term changes in gene expression likely result from epigenetic changes such as DNA methylation and histone modifications (Alyamani and Murgatroyd, 2018; Buschdorf and Meaney, 2016; Fogelman and Canli, 2019; Lux, 2018; Vaiserman, 2015).

Activation of the neuroendocrine stress axis during critical periods of development permanently alters endocrine function, behavior, and disrupts metabolic pathways that may predispose individuals to metabolic disorders, obesity and type 2 diabetes. Similar findings in amphibians support that the developmental role for GCs to 'program' the phenotype is phylogenetically ancient and evolutionarily conserved. Amphibians and other nonmammalian vertebrates are important model organisms for elucidating the effects of exposure to stressors during postembryonic development, the molecular mechanisms for stress hormone actions in early development, and the consequences of these actions for later life phenotypic expression.

CRediT authorship contribution statement

Robert J. Denver: sole author of this manuscript.

Declaration of competing interest

The author, Robert John Denver, declares that he has no conflict of interest.

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