CHAPTER SEVEN

Neuroendocrinology of Amphibian Metamorphosis

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Abstract

The timing of metamorphosis is a central amphibian life history trait and is controlled by the interplay of developmental progression, body size and condition, and environmental signals. These different processes and signals are integrated by the neuroendocrine system to regulate production of hormones by the thyroid gland. Thyroid hormone (TH) is the primary morphogen controlling metamorphosis, while corticosteroids (CSs) produced by the interrenal glands synergize with TH to promote metamorphic changes. The actions of TH are modulated by monodeiodinase enzymes expressed in TH target tissues. CSs act by sensitizing tissues to the actions of TH via the upregulation of TH receptors and monodeiodinases. The increase in thyroid gland activity during metamorphosis is controlled by the hypothalamus and pituitary gland. The hypothalamic-pituitary-thyroid and hypothalamic-pituitary-interrenal axes are regulated at multiple levels. Hypothalamic corticotropin-releasing factor (CRF) functions as a common, central regulator of pituitary thyroid-stimulating hormone (TSH) and adrenocorticotropic hormone (ACTH) secretion in tadpoles. CRF neurons transduce the signals of environmental change (e.g., pond drying, resource availability, etc.) on metamorphic timing by regulating TSH and ACTH secretion, and consequently the production of TH and CS.
1. HORMONAL CONTROL OF METAMORPHOSIS

Metamorphosis is a stage of the amphibian life cycle that is characterized by dramatic morphological transformation accompanied by a transition in ecological niche and behavioral mode. Hormones orchestrate the diverse morphological and physiological changes that occur during metamorphosis, and also function as mediators of environmental effects on development. A striking characteristic of amphibian metamorphosis is that a single signaling molecule produced by the thyroid gland (thyroid hormone, TH) can orchestrate the entire suite of molecular, biochemical, and morphological changes. TH is required for amphibian metamorphosis (Brown & Cai, 2007); the hormone initiates gene expression programs in diverse tissues that lead to cell proliferation, death, differentiation, or migration (Brown & Cai, 2007). Hormones produced by the anterior pituitary gland and the interrenal glands (amphibian homologs of the mammalian adrenal cortex; corticosteroids, CSs) influence the rate of metamorphosis by controlling TH production and action on target tissues. Neurohormones produced in the hypothalamus control hormone biosynthesis and secretion by the pituitary gland, and the hypothalamus mediates the interaction between the external and internal environments, and the production of hormones that control metamorphosis.

1.1. Thyroid hormone

The thyroid gland develops early in the amphibian embryo and matures functionally at the time of hatching when it separates into two distinct lobes and is essentially completely developed by the onset of metamorphosis (Dodd & Dodd, 1976; Kaye, 1959, 1961; Nieuwkoop & Faber, 1956; Regard, Taurog, & Nakashima, 1978; Saxen, Saxen, Toivonen, & Salimaki, 1957a, 1957b). Thyroid activity increases markedly during prometamorphosis, peaks at metamorphic climax, and declines thereafter to reach an “adult” level of activity (Dodd & Dodd, 1976; Kaye, 1959, 1960; Kikuyama, Kawamura, Tanaka, & Yamamoto, 1993; Regard et al., 1978). The major product of the amphibian thyroid gland is 3,5,3′,5′-tetraiodothyronine (thyroxine; T₄) with minor amounts of 3,5,3′-triiodothyronine (T₃) produced (Buscaglia, Leloup, & De Luze, 1985; Rosenkilde, 1978). Coincident with measures of thyroid activity, plasma concentration and whole-body content of T₃ and T₄ increase throughout prometamorphosis and peak at metamorphic climax (Denver,
As in other vertebrates, $T_3$ has greater biological activity than $T_4$ in amphibia owing to the TH receptors (TRs) having 10–15 times greater affinity for $T_3$ than for $T_4$ (Frieden, 1981; Leonard & Visser, 1986; Lindsay, Buettner, Wimberly, & Pittman, 1967; Oppenheimer, Schwartz, & Strait, 1995; Rosenkilde, 1978; Wahlborg, Bright, & Frieden, 1964; White & Nicoll, 1981).

### 1.1.1 TH metabolism

An important point of control of TH bioactivity is at the target tissues, where monodeiodinase enzymes convert $T_4$ to $T_3$, or inactivate $T_4$ and $T_3$ (Fig. 7.1). The monodeiodinases catalyze two basic reactions: a 5$\prime$-monodeiodination (outer ring) that results in bioactivation; and a 5-monodeiodination (inner ring) that results in bioinactivation of the substrate, $T_4$ or $T_3$. There are three types of vertebrate deiodinases (types I, II, and III) that differ in their substrate specificity, kinetics, and sensitivity to inhibitors. Type I catalyzes both 5- and 5$\prime$-, type II 5$\prime$-, and type III 5-deiodination (St Germain, Galton, & Hernandez, 2009). Type II and type III, but not type I enzyme activities have been detected in tadpole tissues, and although frogs have a type I (Dio1) gene, little is known about its expression or function (Becker, Stephens, Davey, Schneider, & Galton, 1997; Dubois et al., 2006; Kuiper et al., 2006).

Three deiodinase genes have been isolated in amphibian species (Brown, 2005). The Dio2 (type II) and Dio3 (type III) genes exhibit tissue-specific and developmental stage-specific expression patterns (Becker et al., 1997; Brown, 2005; Cai & Brown, 2004). The expression patterns correlate with the asynchronous tissue morphogenesis, and the roles that the deiodinases play in modulating intracellular $T_3$ concentration during metamorphosis (Brown, 2005; St Germain et al., 2009). In many cells, both enzymes may be expressed, and the relative expression levels may establish a type of push–pull mechanism that regulates intracellular $T_3$ concentration (St Germain et al., 2009). Alternatively, in some tissues, the two genes show different temporal dynamics, leading to hormone inactivation or activation at different developmental stages.

For example, Dio3 mRNA is expressed in several cell types in tadpole tail, but not in tail muscle cells (Berry, Schwartzman, & Brown, 1998); both Dio3 mRNA and 5-deiodinase activity increase during late prometamorphosis (NF stage 59–61) but then decline sharply at metamorphic climax (Brown et al., 1996; St Germain et al., 1994). This pattern of Dio3 expression may protect the tadpole tail, an essential locomotory organ, from premature resorption (Brown, 2005). By contrast, Dio2 expression, which occurs mainly in tail
Figure 7.1 Central and peripheral organization of the thyroid and stress endocrine axes controlling amphibian metamorphosis. A schematic representation of the hypothalamo–pituitary–thyroid (HPT) and hypothalamo–pituitary–adrenal (HPA; stress) axes in amphibian tadpoles, their regulation by input from the external environment, transduction of this input by neural and neuroendocrine pathways, and synergistic interactions among thyroid hormones and corticosteroids in target cells leading to the promotion of metamorphosis. The two endocrine axes are controlled centrally by corticotropin-releasing factor (CRF) which acts on the anterior pituitary gland (AP) to stimulate the release of thyrotropin (TSH) and corticotropin (ACTH). TSH acts on the thyroid gland to stimulate release of thyroxine (T₄) and 3,5,3'–triiodothyronine (T₃). Thyroid hormones are transported in the blood bound by serum binding proteins (transthyretin, TBG, and albumin). ACTH acts on adrenal cortical cells in the interrenal glands to stimulate biosynthesis and
fibroblasts, is undetectable before late prometamorphosis at which time expression increases markedly and is maintained through the end of metamorphosis (Cai & Brown, 2004). This late expression of *Dio2* is hypothesized to generate bioactive T3 at an appropriate developmental stage to accelerate tail resorption. Neither *Dio2* nor *Dio3* mRNAs are expressed in tail muscle cells (Berry et al., 1998; Bonett, Hoopfer, & Denver, 2010); nonmuscle tail cells may inactivate T4 during pre- and prometamorphosis to protect tail muscle cells from apoptosis, and subsequently generate high local concentrations of T3 to promote tail muscle cell apoptosis at metamorphic climax.

Tissue transformations during metamorphosis are asynchronous: some tissues respond early to low plasma concentrations of TH (e.g., hindlimb, brain), while other tissues respond later in development and require high TH concentration (e.g., intestine, tail—discussed above). The expression patterns of the monodiodinase genes may play a key role in establishing tissue competence to respond to the TH signal. For example, for tissues that respond early in metamorphosis to TH like the retina and hindlimb, *Dio2* expression was high during early prometamorphosis but declined at metamorphic climax. The importance of 5′-deiodinase activity for hindlimb development is supported by findings that T4 has no effect on the hindlimb release of glucocorticoids which are transported in the blood bound to corticosteroid-binding globulin. Cellular uptake of T3 and T4 is achieved by organic anion, monocarboxylate, and amino acid transporters; there is also evidence that thyroid hormones may enter cells bound to transthyretin via a receptor-mediated process. Glucocorticoids enter cells by passive diffusion across the plasma membrane. Upon entering the cell, thyroid hormone is bound by cytosolic-binding proteins, some of which (the monodeiodinases) convert the hormone to either active (T3; deiodinases types I and II) or inactive forms (reverse T3 [rT3], diiodothyronine [T2]; deiodinase type III). TRs form heterodimers with RXRs and are bound to DNA in the unliganded form where they actively repress gene transcription. Upon thyroid hormone binding to TR, gene transcription is derepressed and activated. Upon entering the cell, glucocorticoids bind to corticosteroid receptors (glucocorticoid receptor [GR] or mineralocorticoid receptor [MR]) that are located in the cytosol bound to heat shock proteins (the “foldosome”). Hormone binding causes a conformational change in the receptor, the release of heat shock proteins, and dimerization and translocation of receptors to the nucleus where they bind DNA to activate or repress target genes. When cells are exposed to low concentrations of thyroid hormone plus glucocorticoids, genes such as the TRs, deiodinase type 2, and the thyroid hormone-inducible transcription factor *Klf9* are activated in a synergistic manner. This leads to enhanced sensitivity of cells to the actions of thyroid hormone, which accelerates metamorphosis. “+” indicates an increase and “−” indicates a decrease in the regulated variable.
in the presence of the deiodinase inhibitor iopanoic acid (Brown, 2005). Dio2 mRNA expression showed a progressive decline in the brain throughout metamorphosis, while brain Dio3 mRNA increased during late prometamorphosis and metamorphic climax (Hogan, Crump, Duarte, Lean, & Trudeau, 2007). TH induces cell proliferation in the early prometamorphic tadpole brain, but cells of the neurogenic zone become refractory to TH action on cell proliferation as metamorphic climax approaches, which may be explained by the temporal patterns of Dio2 and Dio3 expression and actions (Cai & Brown, 2004; Denver, Scanlan, & Furlow, 2009).

TH regulates the expression of the Dio2 and Dio3 genes. Dio3 appears to be a direct T3 response gene based on its T3 response kinetics and the resistance of its upregulation to protein synthesis inhibition (Becker, Schneider, Davey, & Galton, 1995; Das, Heimeier, Buchholz, & Shi, 2009; Denver, Pavgi, & Shi, 1997; Hogan et al., 2007; Kawahara, Gohda, & Hikosaka, 1999; St Germain et al., 1994; Wang & Brown, 1993). TH positively regulates 5’-deiodinase activity and Dio2 mRNA in tadpoles (Brown, 2005; Buscaglia et al., 1985; Hogan et al., 2007). However, unlike Dio3, which is an early T3 response gene, Dio2 exhibits delayed response kinetics; the gene was not isolated in screens for early response, direct TR target genes in Xenopus tadpole tissues (Bonett et al., 2010; Brown, 2005; Buchholz, Heimeier, Das, Washington, & Shi, 2007; Das et al., 2006). The dependence of Dio2 expression on TH may vary among tissues. Tissues in which cell proliferation occurs as an early response to TH constitutively express relatively high levels of Dio2 (e.g., neurogenic zones of the brain and spinal cord, limb buds); whereas, in tissues that transform later, Dio2 is upregulated by TH (Cai & Brown, 2004). Treatment of early prometamorphic tadpoles with T4 can induce cell proliferation in these tissues, which can be blocked by the deiodinase inhibitor iopanoic acid (Cai & Brown, 2004). Dio2 mRNA in brain and spinal cord declines at metamorphic climax, as does TH-dependent cell proliferation (Cai & Brown, 2004; Denver et al., 2009). While the decline in Dio2 mRNA may be permissive for the reduction in TH-dependent cell proliferation in the brain, it does not alone explain why the brain becomes refractory to TH action since treatment with T3 could not increase cell proliferation (which normally declines) as the animals approach metamorphic climax (Denver et al., 2009). The decline in cell proliferation is likely due to processes, likely under TH control, that lead to a reduction in the stem cell/progenitor pool in the ventricular/subventricular zones of the tadpole brain. Dio2 expression then appears in late-responding tissues such as the intestine, tail, and anterior pituitary and may be induced at
this time by rising plasma titers of TH (Cai & Brown, 2004; Huang, Cai, Remo, & Brown, 2001; Manzon & Denver, 2004; discussed more below). Physiological roles for tissue monodeiodinases in the timing of metamorphosis are supported by experiments with iopanoic acid and transgenesis over-expression of Dio3 (Becker et al., 1997; Buscaglia et al., 1985; Cai & Brown, 2004; Galton, 1989; Huang et al., 2001; Huang, Marsh-Armstrong, & Brown, 1999; Marsh-Armstrong, Huang, Remo, Liu, & Brown, 1999).

1.1.2 Plasma TH transport proteins

Thyroxine synthesized by thyroid follicular cells diffuses into the bloodstream where it is reversibly bound by plasma proteins that transport the hormone from the site of production to its target tissues (Fig. 7.1). Two plasma-binding proteins that bind T₄ and T₃ with moderate to high affinities have been identified in vertebrates. Thyroxine-binding globulin (TBG) binds T₄ with high affinity and low capacity but is found only in large, eutherian mammals (Power et al., 2000). Transthyretin (TTR; also known as prealbumin) is found in all vertebrates and it binds T₄ with moderate affinity and intermediate capacity. Both TBG and TTRs can also bind T₃, although in most cases with 10 times lower affinity than T₄ (Power et al., 2000); although, the situation in amphibia is the reverse, where TTR binds T₃ with greater affinity than T₄ (Yamauchi, Kasahara, Hayashi, & Horiuchi, 1993; Yamauchi, Nakajima, Hayashi, & Hara, 1999; Yamauchi, Prapunpoj, & Richardson, 2000; Yamauchi et al., 1998). The two primary sites for TTR expression in vertebrates are the liver and the choroid plexus (although it is expressed at other sites; Power et al., 2000). In amphibians, TTR is expressed primarily in the liver (Power et al., 2000). An essential function of TTR is its interaction with retinol-binding protein, which acts as a carrier for all-trans-retinol in the blood. The functional significance of this interaction is not known, but it is intriguing that T₃ and 9-cis-retinoic acid (which is a metabolite of all-trans-retinol) serve as ligands for the TR–retinoid X receptor (RXR) heterocomplex that regulates TH target genes. Serum albumin also binds T₃ and T₄ in many species with low affinity and high capacity, and Power et al. (2000) suggested that albumin might be the principal T₄-binding protein in amphibia.

Circulating TTR is present in tadpoles during premetamorphosis and prometamorphosis when thyroid activity is increasing, but declines at metamorphic climax (Prapunpoj, Yamauchi, Nishiyama, Richardson, & Schreiber, 2000; Yamauchi et al., 2000, 1998). The free hormone hypothesis (Ekins, 1990; Mendel, 1989) leads to the prediction that TTR
during pre- and early prometamorphosis serves to reduce the free fraction of TH in blood thus limiting bioavailability. Conversely, hormone-binding proteins can serve as a reservoir for hormone in the blood; TTR could therefore help to sustain increasing plasma TH concentrations prior to the acceleration of thyroid gland activity induced by rising plasma thyroid-stimulating hormone (TSH) titers. The TTR concentration in the blood declines at metamorphic climax when plasma TH concentration is maximal. The continued rise in TH synthesis by the thyroid gland, paired with a decline in TTR, could result in an increase in the free hormone fraction in the blood. At the same time, the rate of clearance of T3 from the circulation would likely increase. However, because the thyroid synthetic rate is high at metamorphic climax, total plasma T3 concentration continues to rise. Thus, one would predict that, not only does the hormone production rate increases at metamorphic climax but also does the proportional availability of T3 to the target tissues. To my knowledge, T3 or T4 clearance rates have not been calculated in tadpoles at different stages of development. Based on TTR expression profiles, one would predict that clearance rates would be lower during prometamorphosis compared with premetamorphosis or metamorphic climax. Further, given the lower affinity of TTR for T4 compared with T3, one would predict that the clearance rate for T4 would be higher than T3.

1.1.3 Membrane TH transporters and cytosolic thyroid hormone-binding proteins

Tadpole cells have the capacity to actively take up TH (see Krain & Denver, 2004), and this activity may be regulated during metamorphosis. Saturable, carrier-mediated uptake of THs has been demonstrated in tadpole RBCs (Galton, Stgermain, & Whittemore, 1986; Murata & Yamauchi, 2005; Yamauchi, Horiuchi, Koya, & Takikawa, 1989). The genes that encode TH transport proteins could be the important loci for the modulation of the timing of metamorphosis.

There are three general classes of proteins that allow for active uptake of TH by cells: the organic anion transporters (OATC), monocarboxylate transporters (MCT), and the L-amino acid permeases (LAT) (Friesema, Jansen, Milici, & Visser, 2005; Jansen, Friesema, Milici, & Visser, 2005; Ritchie, Peter, Shi, & Taylor, 1999; Ritchie et al., 2003; Visser, Frieserna, Jansen, & Visser, 2008). Orthologs of oatc, mct and lat genes have been isolated from frogs and patterns of expression throughout metamorphosis have been described (Connors, Korte, Anderson, & Degitz, 2010;
Only the amino acid permeases have so far received attention with regard to their function during tadpole metamorphosis. The T₃-inducible gene *iu12* from *Xenopus laevis* intestine (Liang et al., 1997; Shi & Brown, 1993) encodes a subunit of a heterodimeric amino acid permease complex (System L; Ritchie et al., 2003; Torrents et al., 1998). This permease complex efficiently transports T₃ and T₄ when expressed in the *Xenopus* oocyte expression system but is inhibited by reverse T₃ (Ritchie et al., 1999). Overexpression of System L in *Xenopus* oocytes increased cytoplasmic and nuclear delivery of THs from the external medium and enhanced transcriptional activation by TRs (Ritchie, Hayashi, Shi, & Taylor, 2002). By contrast, blocking endogenous System L activity in mammalian cells reduced both TH uptake and TR function (Ritchie et al., 2003). The fact that *iu12* is a T₃-inducible gene suggests that it could play a role in mediating T₃ uptake by cells during tadpole metamorphosis (Liang et al., 1997).

Upon entering cells, THs may bind to a series of intracellular-binding proteins, termed cytoplasmic TH-binding proteins (CTHBPs) that represent several classes of multifunctional proteins. These proteins may have a variety of enzymatic activities; for example, three genes isolated from *X. laevis* code for (1) a cytosolic aldehyde dehydrogenase which catalyzes the formation of retinoic acid (Yamauchi & Tata, 1994), (2) an M2 pyruvate kinase (Shi, Liang, Parkison, & Cheng, 1994), and (3) protein disulfide isomerase (PDI; induced by T₃ in tadpole brain; Denver et al., 1997), which catalyzes disulfide bond formation and human PDI has been shown to bind TH with high affinity (Cheng et al., 1987; Yamauchi et al., 1987). These CTHBPs may transport THs within the cytoplasm to the nucleus to bind to TRs, or alternatively, they may serve as chelators to limit the cellular free TH concentration (Shi, 2000). It is also possible that TH may regulate the enzymatic activity of these proteins (Ashizawa & Cheng, 1992).

### 1.2. Corticosteroids

#### 1.2.1 CS production during metamorphosis

In addition to TH, CSs, the primary vertebrate stress hormones, play important roles in amphibian metamorphosis. Like TH, the production of CS changes with development and likely reflects the functional maturation of the hypothalamo–hypophyseal–interrenal axis. The major CS produced by the amphibian interrenal glands are corticosterone (CORT) and aldosterone (ALDO) (Carstensen, Burgers, & Li, 1961; Macchi & Phillips, 1966).
In most species studied, the plasma concentration and tissue content of CORT and ALDO increase during late prometamorphosis/metamorphic climax, more or less in parallel with the rise in TH production (Carr & Norris, 1988; Denver, 1998a; Glennemeier & Denver, 2002a; Jaffe, 1981; Jolivet-Jaudet & Leloup-Hatey, 1984; Kikuyama, Suzuki, & Iwamuro, 1986; Kloas, Reinecke, & Hanke, 1997; Krain & Denver, 2004; Krug, Honn, Battista, & Nicoll, 1983; Niinuma et al., 1989). The majority of these studies showed low to nondetectable CS during premetamorphosis and a marked increase at metamorphic climax. The only exception is in *X. laevis* where whole-body CS content may be highest during premetamorphosis (Kloas et al., 1997); although, there is a secondary, lower peak at metamorphic climax (Glennemeier & Denver, 2002a). CSs, being lipophilic, are transported in blood bound to plasma proteins. Recently, binding properties of a putative corticosteroid-binding globulin (CBG) were described in the serum of an amphibian (*Ambystoma tigrinum*) (Orchinik, Matthews, & Gasser, 2000). However, to my knowledge, the expression of CBG has not been studied in amphibians.

### 1.2.2 CS actions during metamorphosis

CSs exert complex effects on tadpole growth and development. Depending on the animal’s developmental stage and TH status, CS can accelerate or decelerate metamorphosis. If elevated during premetamorphosis, CS typically inhibit tadpole growth and slow development (Belden, Moore, Wingfield, & Blaustein, 2005; Darras et al., 2002; Frieden & Naile, 1955; Glennemeier & Denver, 2002b; Gray & Janssens, 1990; Hayes, 1995; Hayes, Chan, & Licht, 1993; Hu, Crespi, & Denver, 2008; Kobayashi, 1958; Wright et al., 1994).

However, CS have been found to accelerate TH-induced and spontaneous metamorphosis (Darras et al., 2002; Frieden & Naile, 1955; Gray & Janssens, 1990; Hayes, 1995; Kikuyama et al., 1993, 1983; Kuhn, De Groef, Grommen, Van der Geyten, & Darras, 2004; Kuhn, De Groef, Van der Geyten, & Darras, 2005; Wright et al., 1994). Taken together, the findings suggest that elevated CS (e.g., in response to environmental stressors) during premetamorphosis retard growth and slow development, while increased CS during prometamorphosis accelerate metamorphosis. The mechanisms of CS inhibition of growth in tadpoles have not been investigated, but based on studies in mammals, these actions could manifest at multiple levels that likely include diverse catabolic actions (Sapolsky,
Romero, & Munck, 2000) and perhaps decreased anterior pituitary growth hormone (GH) biosynthesis (Harvey, Scanes, & Daughaday, 1995).

Recent findings suggest several molecular mechanisms for the positive interactions between CS and TH signaling in the acceleration of metamorphosis. CSs enhance TH bioactivity, increasing expression of TR and monodeiodinase genes. They increase maximal nuclear-binding capacity for T₃ (Kikuyama et al., 1993; Niki, Yoshizato, & Kikuyama, 1981; Suzuki & Kikuyama, 1983), which is paralleled by the upregulation of tr mRNAs in X. laevis tail and in frog cell lines; this occurs in a synergistic manner with low or subthreshold doses of TH plus CORT causing superinduction of TRs (Bonett et al., 2010). CORT also increased 5'-deiodinase activity and Dio2 mRNA in tadpoles, thereby increasing T₃ in target tissues (Bonett et al., 2010; Darras et al., 2002; Galton, 1990; Kuhn et al., 2005). Notably, the action of CORT on Dio2 was synergistic with T₃ in tadpole tail (Bonett et al., 2010).

Direct TH target genes may also be synergistically regulated by T₃ and CS through mechanisms that are not directly or immediately dependent on increased TRs or deiodinases (i.e., direct synergy between TRs and CS receptors at the target gene). For example, Krüppel-like factor 9 (Klf9; also known as BTEB1), a direct T₃ target gene, is induced by CORT (Bonett, Hu, Bagamasbad, & Denver, 2009) and is superinduced in tadpole tissues with rapid kinetics by combined treatment with T₃ plus CORT (P. Bagamasbad, R.M. Bonett, and R.J. Denver, unpublished data). Other genes are synergistically regulated by TH and CS, which could explain the mechanism by which these two hormones cooperate to accelerate metamorphosis (Kulkarni et al., 2012).

2. NEUROENDOCRINE CONTROL OF METAMORPHOSIS

The vertebrate neuroendocrine system is comprised of the hypothalamus and the pituitary gland (Fig. 7.1). The importance of hypothalamic control of metamorphosis has long been recognized (Denver, 1996; Kikuyama et al., 1993). The pituitary hormones that control TH and CS production, TSH and adrenocorticotropic hormone (ACTH), respectively, are primarily under stimulatory hypothalamic control in amphibians (Denver, 1996). The neuroendocrine system serves as an interface between the central nervous system and the endocrine system, and transduces signals derived from the external and internal environments into appropriate physiological/developmental responses.
2.1. Thyroid-stimulating hormone

Hypophysectomy of tadpoles arrests the development of the thyroid gland and leads to metamorphic stasis that is reversed by injecting TSH (Dodd & Dodd, 1976; Regard & Mauchamp, 1971, 1973). The rate of thyroid gland growth and TH production in the tadpole is coordinate with the development of the pituitary and the production of TSH (Buckbinder & Brown, 1993; Denver, 1996; Dodd & Dodd, 1976; Kaye, 1961; Korte et al., 2011; Manzon & Denver, 2004; Okada et al., 2009, 2004). The amphibian thyroid gland develops sensitivity to TSH just before hatching (Kaye, 1961).

Pituitary expression of tshβ mRNA and plasma TSH concentration increases throughout metamorphosis (Buckbinder & Brown, 1993; Manzon & Denver, 2004; Okada et al., 2000). Pituitary tshβ mRNA levels rise from premetamorphosis to peak values during late prometamorphosis/metamorphic climax (Buckbinder & Brown, 1993; Manzon & Denver, 2004; Okada et al., 2000). Korte et al. (2011) used a homologous radioimmunoassay to show that changes in plasma and pituitary TSH in Xenopus species throughout metamorphosis paralleled changes in pituitary tshβ mRNA. Thus, TSH biosynthesis is coordinate with thyroid gland development and hormone secretion, and the stimulatory action of pituitary TSH is necessary for thyroid gland growth and hormone biosynthesis.

The tripeptide amide thyrotropin-releasing hormone (TRH), which is the primary regulator of TSH release in adult mammals, is inactive on tadpole TSH secretion, although the thr gene is expressed in the brain and pituitary of amphibians (Denver, 1996; Denver & Licht, 1989; Kikuyama et al., 1993; Manzon & Denver, 2004; Norris & Dent, 1989; Okada et al., 2004), and TRH can stimulate TSH release in adult frogs (Denver, 2009a; Galas et al., 2009). Many studies now support that the secretion of TSH by the tadpole pituitary gland is under stimulatory control by corticotropin-releasing factor (CRF) and related peptides (e.g., sauvagine, urocortins; Denver, 2009b, 2009c). CRF-like peptides regulate neuroendocrine, autonomic, and behavioral responses to physical and emotional stress (Aguilera, 1998; Yao & Denver, 2007).

CRF was named for its role in inducing release of pituitary ACTH in mammals, a role that is shared in amphibia (Vale, Vaughan, & Perrin, 1997). Shortly after its discovery in 1981, CRF was discovered to be a potent stimulator of the thyroid axis in larval amphibians and other nonmammalian vertebrates (De Groef, Van der Geyten, Darras, & Kuhn, 2006; Denver, 1999, 2009b, 2009c). Treatment of amphibian pituitary explants or primary pituitary cells with CRF-like peptides stimulated TSH release (De Groef
et al., 2006; Denver, 2009b, 2009c; Okada et al., 2009), and injections of CRF-like peptides elevated whole-body TH content in tadpoles of several species (Boorse & Denver, 2004; Denver, 1993, 1997; Gancedo et al., 1992).

Commensurate with their positive actions on tadpole thyroid activity, CRF-like peptides have been shown to accelerate tadpole metamorphosis (Boorse & Denver, 2002; Denver, 1993, 1997; Gancedo et al., 1992; Miranda, Affanni, & Paz, 2000). Conversely, blocking endogenous CRF by passive immunization with CRF antiserum, or by injection of the CRF receptor antagonist α-helical CRF(9–41) slowed spontaneous metamorphosis, or blocked simulated pond drying-induced metamorphosis (Denver, 1997). Further, hypothalamic of mRNA and peptide content increased during spontaneous metamorphosis (Denver, 2009b), and hypothalamic CRF peptide content was increased in spadefoot toad tadpoles that accelerated metamorphosis in response to simulated pond drying (Denver, 1997). Kulkarni, Singamsetty, and Buchholz (2010) recently showed that CRF accelerates development of the direct developing frog Eleutherodactylus coqui. Because CRF is a stress neurohormone, endogenous CRF may participate in environmentally induced (stress-induced) metamorphosis (Boorse & Denver, 2004; Denver, 1997).

Work from Sakae Kikuyama’s laboratory found that a majority of the TSH-releasing activity of tadpole and adult frog hypothalamic extracts on dispersed adult pituitary cells can be blocked by coincubation with the CRF receptor antagonist α-helical CRF(9–41) (Ito et al., 2004; Okada et al., 2009). These findings suggest that a significant proportion of TSH-releasing activity in the amphibian hypothalamus is contributed by CRF-like peptides. They also suggest that other factors could be involved in the regulation of TSH, or that α-helical CRF(9–41) may have only partial antagonist activity in amphibia as has been found in mammals (Rivier, Rivier, & Vale, 1984).

CRF actions are mediated by two G protein-coupled receptors (CRF1 and CRF2; Dautzenberg & Hauger, 2002) and are modulated by a secreted-binding protein (CRF-BP; Seasholtz, Valverde, & Denver, 2002). The action of CRF-like peptides on TSH release in the tadpole is mediated by the CRF2 receptor expressed in thyrotropes (Okada et al., 2007, 2009); whereas, ACTH release may be controlled by the CRF1 receptor in amphibians as it is in mammals (De Groef, Geris, et al., 2003; De Groef, Goris, Arckens, Kuhn, & Darras, 2003; Okada et al., 2009; Tonon et al., 1986; Van Pett et al., 2000). In X. laevis, crf1 mRNA was expressed during premetamorphosis and its level increased during prometamorphosis, reaching
a plateau through metamorphic climax (Manzon & Denver, 2004). In contrast, mRNA for the $\alpha f_2$ was very low during pre- and early prometamorphosis, but increased dramatically during late prometamorphosis and metamorphic climax. The expression of the $\alpha f_2$ in the tadpole pituitary paralleled the increase in sensitivity of the pituitary to CRF-like peptides during metamorphosis (Fig. 7.2; Kaneko et al., 2005). These findings support the hypothesis that the competence of tadpole pituitary thyrotropes to respond to hypothalamic CRF depends on the upregulation of the CRF$_2$ receptor during late prometamorphosis.

Figure 7.2  Pituitary expression of the corticotropin-releasing factor (CRF) receptor type 1 (CRF$_1$) and type 2 (CRF$_2$) show distinct patterns during tadpole metamorphosis. (A) Semiquantitative RT-PCR analysis of $crf_1$ and $crf_2$ mRNAs in X. laevis tadpole pituitary. The $rpl8$ is a housekeeping gene whose expression did not change during development. (B) Quantitation of semiquantitative RT-PCR analysis of $crf_1$ and $crf_2$ mRNAs in X. laevis tadpole pituitary. $crf_1$ and $crf_2$ mRNA levels were normalized to $rpl8$ mRNA (modified from Manzon & Denver, 2004). (C) Thyroid-stimulating hormone (TSH) secretion by dispersed tadpole or frog pituitary cells treated with vehicle or frog CRF (100 nM) for 24 h. Data were derived from Kaneko et al. (2005), Fig. 4.
2.1.1 Feedback regulation of TSH

Negative feedback by TH on the hypothalamus and pituitary plays a central role in thyroid homeostasis in all adult vertebrates that have been studied including frogs (Fig. 7.1; Jacobs & Kuhn, 1992; Kaneko et al., 2005). The discovery of a sustained rise in TSH production and thyroid activity during tadpole metamorphosis led Etkin (1968) to hypothesize that negative feedback on pituitary TSH does not develop until metamorphic climax. Huang et al. (2001) proposed that the onset of negative feedback at metamorphic climax was coincident with the expression of Dio2 in the tadpole pituitary. However, many investigators have found that negative feedback by TH on TSH is active in the premetamorphic and early prometamorphic tadpole. For example, treatment of premetamorphic tadpoles with goitrogens caused enlargement of the thyroid gland and degranulation of pituitary thyrotropes, while replacement with T4 reversed the effects, suggesting that negative feedback was functional in the premetamorphic tadpole (Dodd & Dodd, 1976; Goos, 1968, 1978; Goos, Deknecht, & Devries, 1968). Further, goitrogen treatment of premetamorphic tadpoles caused a dramatic elevation in tshβ mRNA (Buckbinder & Brown, 1993; Huang et al., 2001).

Physiological concentrations of T4 or T3 can act directly on pituitary explants of X. laevis tadpoles throughout metamorphosis to suppress tshβ mRNA expression and TSH secretion (Manzon & Denver, 2004; Sternberg et al., 2011). Pituitary sensitivity to negative feedback by TH may decline slightly during late metamorphosis and metamorphic climax, perhaps due to the upregulation of pituitary Dio3 at this time (Manzon & Denver, 2004; Sternberg et al., 2011). Kaneko et al. (2005) found that CRF-induced TSH release by bullfrog primary pituitary cells was suppressed by T3 throughout metamorphosis. Taken together, these findings support that negative feedback at the level of the pituitary is active in the premetamorphic and early prometamorphic tadpole, which does not support Etkin’s hypothesis and contradicts Huang et al. (2001).

Deiodinase type 2 plays an important role in TH-negative feedback on TSH in mammals (Schneider et al., 2001; St Germain, Hernandez, Schneider, & Galton, 2005). The Dio2 gene is expressed in the tadpole from early prometamorphosis and shows a progressive increase during metamorphosis, reaching a maximum by NF stage 59 (Manzon & Denver, 2004). This supports the findings discussed above that T4, likely through conversion to T3, exerts negative feedback on TSH throughout tadpole metamorphosis. The downregulation of TSH expression by T4 suggests that 5'-deiodinase activity is either present in the pituitary throughout prometamorphosis or
the conversion of $T_4$ to $T_3$ is not required for negative feedback. TH receptor $\beta$ is required for transcriptional repression of the $tsh\beta$ and $trh$ genes in mammals (Flamant & Samarut, 2003; Guissouma, Dupre, & Demeneix, 2005). $tr\beta$ mRNA increased throughout metamorphosis in the tadpole pituitary (Manzon & Denver, 2004).

Despite the presence of functional negative feedback in the prometamorphic tadpole, TSH production shows a progressive increase throughout metamorphosis reaching a peak at metamorphic climax. Hypothalamic neurosecretory neurons and the median eminence, the structure necessary for the delivery of neurohormones to the pituitary, develop during prometamorphosis under the influence of TH (Denver, 1998b). The expression of neuropeptide receptors by anterior pituitary cells, and the responsiveness of these cells to secretagogues increases during metamorphosis (Kaneko et al., 2005; Manzon & Denver, 2004). Etkin (1968) first proposed that the maturation of the hypothalamus, median eminence, and pituitary under the influence of TH is responsible for the sustained rise in plasma TH concentration that drives metamorphosis. Thus, combined with a slight decrease in the sensitivity of the pituitary to negative feedback at metamorphic climax, the hypothalamic drive for TSH production may be sufficient to overcome negative feedback exerted by the elevated plasma TH concentration at this time. Negative feedback is likely to be physiologically important for limiting TSH secretion once the system has matured, and perhaps during maturation of the neuroendocrine system; that is, the coordination of morphogenesis may require the temperance of TSH expression by TH throughout metamorphosis. However, the sustained rise in thyroid activity during metamorphosis is likely to be due primarily to the maturational effects of TH on the CNS (and perhaps the pituitary) rather than the absence of negative feedback. The relatively lower levels of pituitary $Dio2$ and $TR\beta$ expression during early prometamorphosis might be permissive for the sustained rise in TSH during prometamorphosis.

2.2. Adrenocorticotrophic hormone

Expression of proopiomelanocortin mRNA in the anterior pituitary is low in premetamorphic tadpoles and increases during prometamorphosis peaking at metamorphic climax (Aida, Iwamuro, Miura, & Kikuyama, 1999). To my knowledge, there have been no direct measures of ACTH during tadpole development. Functional ACTH receptors are expressed
by tadpole interrenal glands prior to the onset of metamorphosis (Glennemeier & Denver, 2002a). Premetamorphic tadpoles are capable of mounting a CORT response following exposure to a physical stressor (shaking/confine stressor; Glennemeier & Denver, 2002a), which suggests that functional maturation of the hypothalamo–hypophyseal–interrenal axis occurs prior to metamorphosis (by contrast to the tadpole hypothalamo–hypophyseal–thyroid axis, which matures during prometamorphosis). The early functional maturation of the hypothalamo–hypophyseal–interrenal axis is reflected in the earlier expression of the CRF<sub>1</sub> receptor (expressed on corticotropes; expression at NF stage 52) compared with the CRF<sub>2</sub> receptor (expressed on thyrotropes; expression at NF stage 57) (Manzon & Denver, 2004; Okada et al., 2009; Fig. 7.2). The early maturation of the hypothalamo–hypophyseal–interrenal axis provides for environmental stressors to elevate endogenous CS production in premetamorphic tadpoles, which can have consequences for tadpole growth and development.

Compared with TSH, much less is known about how the hypothalamus controls ACTH secretion in amphibia. CRF and arginine vasopressin (AVT is the amphibian hormone) have been shown to stimulate ACTH secretion by cultured adult frog pituitaries (Tonon et al., 1986).

### 2.3. Growth hormone and prolactin

A central prediction of Etkin’s model for the endocrine control of tadpole metamorphosis (Etkin, 1968) was that the metamorphic actions of TH were balanced by the inhibitory actions of the pituitary hormone prolactin (PRL). Etkin proposed that in the premetamorphic tadpole, PRL secretion was high, but declined at metamorphic climax. This prediction was based in large part on the inhibitory effects of injecting mammalian PRLs on metamorphosis (White & Nicoll, 1981), which led some investigators to suggest that PRL exerted a juvenilizing action in amphibian larvae similar to juvenile hormone in insects (Bern, Nicoll, & Strohman, 1967; Etkin & Gona, 1967).

The early studies that led to the development of the Etkin model have been extensively reviewed (Denver, 1996; Dodd & Dodd, 1976; Kaltenbach, 1996; Kikuyama et al., 1993; White & Nicoll, 1981). Work using mostly mammalian PRL or GH preparations suggested different roles for these hormones, with PRL enhancing larval growth and blocking the actions of TH on metamorphosis, and GH stimulating postmetamorphic growth as the hormone does in most vertebrates (Denver, 1996; Takada &
Kasai, 2003). A role for GH in regulating body growth in amphibia as in other vertebrates (Harvey et al., 1995) has been demonstrated by many studies in which GH was injected into tadpoles or frogs (Denver, 1996; Kikuyama et al., 1993; White & Nicoll, 1981) and more recently through transgenic overexpression of GH in X. laevis (Huang & Brown, 2000a).

In contrast to GH, a role for PRL in regulating tadpole growth and metamorphosis continues to be controversial (Huang & Brown, 2000b). Early work supporting that PRL inhibited metamorphosis and stimulated larval growth was conducted with mammalian PRL (and GH) preparations. These studies clearly showed that functional receptors for PRL/GH are expressed in amphibian tissues and their activation can both promote tadpole growth and block T3-induced metamorphosis; the latter action likely through the prevention of TRβ autoinduction (Tata, Baker, Machuca, Rabelo, & Yamauchi, 1993). Further, injection of purified frog PRL had similar effects on tadpole growth and development as mammalian PRL (Kikuyama et al., 1993).

One can argue that the effects of exogenous hormones may represent pharmacological rather than physiological actions. However, it is noteworthy that blockade of endogenous PRL by passive immunization accelerated metamorphic changes, which supports a physiological role for the endogenous hormone (Denver, 1996; Kikuyama et al., 1993). Etkin (1968) proposed that larval growth and metamorphosis is controlled by a balance between TH and PRL, and that the two should show an inverse relationship in their blood concentrations at metamorphic climax. The rise in circulating concentrations of TH during prometamorphosis and climax have been confirmed (see above). However, circulating concentrations of PRL and levels of pituitary prl mRNA are low during premetamorphosis and also rise, more or less in parallel with TH, during late prometamorphosis and climax (Buckbinder & Brown, 1993; Clemons & Nicoll, 1977; Niinuma, Yamamoto, Yamamoto, & Kikuyama, 1991; Takahashi et al., 1990; Yamamoto & Kikuyama, 1982), thus contradicting the earlier hypothesis of an inverse relationship of the two hormones (Etkin, 1968). The rise in PRL production tends to occur slightly later than the rise in TSH expression and circulating TH (Buckbinder & Brown, 1993). Similarly, [125I]-PRL binding to kidney membrane fractions was low in premetamorphic bullfrog tadpoles and increased during metamorphic climax (White & Nicoll, 1979). Huang and Brown (2000b) measured PRL receptor mRNA in whole X. laevis tadpole and tail tissue and found increased expression at metamorphic climax. Hasunuma, Yamamoto, and Kikuyama (2004) found that PRL receptor mRNA increased in the tail fin and kidney of bullfrog tadpoles during
metamorphic climax. Taken together, these findings argue against the hypothesis that PRL plays a juvenilizing role in amphibian metamorphosis (Buckbinder & Brown, 1993; Huang & Brown, 2000b). However, Kikuyama et al. (1993) have argued, based on their experiments with passive immunization with antiserum to bullfrog PRL, that low levels of PRL during the premetamorphic/early prometamorphic period might be sufficient to support larval growth and inhibit TH action.

Huang and Brown (2000a, 2000b) created transgenic *X. laevis* tadpoles that overexpressed *X. laevis* GH, *X. laevis* PRL, or ovine PRL. All tadpole tissues expressed the transgenes driven by the simian cytomegalovirus promoter; that is, expression was not restricted to the pituitary gland where the hormones are normally produced. They found that overexpression of GH did not affect the timing of metamorphosis but resulted in larger tadpoles and larger juvenile frogs. Overexpression of frog or ovine PRL had no effect on the timing of most metamorphic changes, but blocked tail resorption in some tadpoles. They concluded that their results disprove the hypothesis that PRL is a juvenile hormone in *X. laevis*. One note of caution in interpreting these findings is that PRL was overexpressed in all tissues throughout development, which could have led to compensatory changes (e.g., receptor desensitization) that masked the physiological roles of the hormone.

The elevation in PRL biosynthesis at metamorphic climax suggests that the hormone could modulate the actions of TH at a time when tissues are undergoing rapid and dramatic transformation (Denver, 1996). Shintani, Nohira, Hikosaka, and Kawahara (2002) showed that PRL and GH induced Dio3 mRNA in tadpole tail, and they proposed that the effects of PRL and GH on metamorphosis may be mediated in part by the tissue-specific regulation of Dio3. PRL secretion is stimulated by TRH (Galas et al., 2009), and it has been hypothesized that, while TRH does not regulate TSH secretion in the tadpole, it plays a role in regulating the rise in PRL production at metamorphic climax (Buckbinder & Brown, 1993; Norris & Dent, 1989; White & Nicoll, 1981). The level of type 2 TRH receptor mRNA in the tadpole pituitary increased through late prometamorphosis and peaked at metamorphic climax (Manzon & Denver, 2004). In mammals, PRL secretion is induced by stressors (Cooke et al., 2004; Soares, Alam, Konno, Ho-Chen, & Ain, 2006). If a similar regulatory relationship exists in amphibia (e.g., see Lorenz, Opitz, Lutz, & Kloas, 2009), then it may be that the activation of neuroendocrine stress pathways during metamorphosis function in the late rise in PRL secretion.
3. ROLE OF THE NEUROENDOCRINE SYSTEM IN MEDIATING ENVIRONMENTAL INFLUENCES ON THE TIMING OF METAMORPHOSIS

The duration of the larval period varies considerably among and within amphibian species. The earliest time for the onset of metamorphosis is established by a genetically determined, species-specific minimum size for transformation. The time that it takes to reach the minimum size is determined in part by growth opportunity in the larval habitat (Werner, 1986; Wilbur & Collins, 1973). The better the resource supply, the earlier that a tadpole can reach its species-specific minimum size for metamorphosis. Variation in the proximate environment establishes trade-offs between growth opportunity and risk of mortality (environmental stress, predation risk, etc.) which ultimately determines the duration that the animal spends as a tadpole. Species that breed in permanent, predictable habitats can have relatively long larval periods (i.e., 3 years or greater); whereas those that breed in unpredictable, ephemeral ponds have short larval periods (as short as 10 days from hatching) (Denver, Boorse, & Glennemeier, 2002). The proximate mechanisms that govern the timing of metamorphosis involve the production, metabolism, and actions of hormones. Competence to respond to environmental signals depends on the development and activity of endocrine glands that produce the hormones that control metamorphosis.

Points of regulation by the environment include the neuroendocrine system, peripheral endocrine organs, hormone transport and metabolism, and hormone action. Thermal, osmotic, and effects related to the gaseous environment may be sensed directly by most or all tissues. Signals generated by other factors, such as photoperiod, resource availability, predator presence, and crowding are integrated by higher brain centers and transduced by the neuroendocrine system into changes in peripheral endocrine gland activity. The activity of the tadpole hypothalamo–pituitary–thyroid axis can be regulated at multiple levels, and thyroid activity determines when larvae enter metamorphosis, and the rate at which metamorphosis progresses. Because the stress hormonal axis is closely linked to the thyroid axis, central nervous stress pathways play a critical role in transducing environmental information and regulating metamorphic timing.

Work of Etkin (1968) suggested that the “clock” that determines the timing of metamorphosis is located in the hypothalamus. He showed that tadpoles in which the pituitary primordium was autotransplanted to the tail...
during embryogenesis grew more rapidly than controls, suggesting that pituitary GH is under inhibitory hypothalamic control; however, the tadpoles failed to metamorphose, supporting that a hypothalamic neurohormone was required to stimulate TSH secretion (Etkin, 1968). Destruction of the preoptic area or surgical removal of the primordium of the posterior hypothalamus (and thus isolation of the pituitary from the brain) prevented metamorphosis (reviewed by Denver, 1996). Investigations of the normal development of the neurosecretory centers of the hypothalamus and the median eminence further support Etkin’s hypothesis (Etkin, 1968).

A striking example of the role of the hypothalamus in controlling metamorphosis, in particular, the role of hypothalamic CRF, comes from studies of desert toad species. The most important environmental variable for a tadpole is water availability, and duration of the aquatic habitat can profoundly influence the rate of metamorphosis in many species. This is especially true for desert amphibians that tend to breed in ephemeral habitats. As discussed earlier, CRF-like peptides control TSH secretion in tadpoles, acting via the CRF$_2$ receptor. Because the secretion of CRF is activated by stressors, CRF plays a central role in mediating a tadpole’s developmental response to a deteriorating larval habitat (e.g., pond drying in the case of the Western spadefoot toad) (Boorse & Denver, 2004; Denver, 1997; Denver, 1998a; Denver, Mirhadi, & Phillips, 1998). The timing of the expression of receptors for neurohormones in the pituitary gland, particularly the CRF$_2$ receptor, could be important in establishing competence of pituitary thyrotropes to respond to stimulation by CRF-like peptides (Manzon & Denver, 2004; Okada et al., 2009).

Other environmental factors that are known to alter the timing of metamorphosis (e.g., food availability, crowding, predation) may also act through the neuroendocrine stress axis. For example, whole-body CORT content was elevated in tadpoles that were food restricted or subjected to high conspecific density, compared to their high resource, low density counterparts (Glennemeier & Denver, 2002b). Both low food and increased density resulted in slowed growth and development in premetamorphic tadpoles, which agrees with other studies showing growth- and development-inhibiting effects of these factors in premetamorphs (but contrast this with prometamorphic animals which accelerate development in response to food restriction or crowding). This slowed growth caused by crowding stress was reversed by treatment of tadpoles with the CORT synthesis inhibitor metyrapone, again suggesting a functional role for the hypothalamo–hypophysial–interrenal axis in
mediating the larval developmental response to environmental conditions (Glennemeier & Denver, 2002b). Hayes (1997) also reported an elevation in whole-body CORT content in tadpoles caused by crowding. By contrast, Belden, Rubbo, Wingfield, and Kiesecker (2007) did not find such a relationship in a mesocosm study. Predation, temperature, photoperiod, or other environmental factors could conceivably work through similar neuroendocrine pathways to exert their effects on larval development. If larvae have a means of detecting the state of environmental conditions, through visual, chemical, or other sensory systems, then the neuroendocrine system is a likely pathway through which developmental responses to the environment can operate.

While the hypothalamus and pituitary gland are required for metamorphosis through their control of thyroid and interrenal gland secretion, other processes occurring at target tissues may influence metamorphic timing. For example, the availability of biologically active TH is regulated within tissues by the monodeiodinases. Buchholz and Hayes (2005) showed that closely related species of spadefoot toads that differ in the duration of their larval periods show strong differences in the tissue content of T3 and T4, and the sensitivity of their tissues to TH. They speculated that these differences might be due to differences in TH uptake into cells and/or TH metabolism. The expression of monodeiodinases enzymes could be modified either directly or indirectly by environmental factors. CS increases 5\'-deiodinase activity, with the result that more of the active hormone T3 is generated. This regulatory relationship suggests that stress and stress hormones could accelerate metamorphosis by upregulating 5\'-deiodinase activity.

Tissue expression of TRs influences sensitivity to the TH signal. TH receptor \( \beta \) is autoinduced in many tissues during metamorphosis, and evidence suggests that this is required to drive metamorphosis (Bagamasbad & Denver, 2011; Laudet, 2011). Hollar, Choi, Grimm, and Buchholz (2011) recently showed that TR expression level is negatively correlated with the duration of the larval period in different species of spadefoot toad; that is, higher TR equals shorter larval period.

Biosynthesis of TRs might be regulated directly or indirectly by environmental factors, which could influence metamorphic timing. Currently, relatively little is known about what factors, either physiological or environmental, regulate nuclear receptor expression in any species (Bagamasbad & Denver, 2011). As for monodeiodinases, evidence suggests that CS can enhance TH action by upregulating TR expression, and so TR
biosynthesis is an additional site where stress and stress hormones may modulate metamorphic timing (Bonett et al., 2010).

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