2.09 Endocrinology of Complex Life Cycles: Amphibians

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Glossary

Adrenocorticotropic hormone (ACTH) Small polypeptide hormone derived from a larger precursor (proopiomelanocortin) produced by the anterior pituitary gland that stimulates the adrenal cortex (interrenal glands in nonmammalian species) to produce corticosteroids (primarily glucocorticoids).

Corticotropin-releasing hormone (CRH) Forty-one-amino-acid polypeptide produced in the hypothalamus and extrahypothalamic sites that stimulates the release of ACTH (all vertebrates studied) and TSH (nonmammalian vertebrates) by the anterior pituitary gland. CRH is a member of a family of peptides of similar size that include the urocorins, urotensin I, and sauvagine. CRH-like peptides play central roles in developmental, behavioral, and physiological responses to stressors.

Growth hormone (GH) Simple polypeptide hormone produced by the anterior pituitary gland that stimulates growth in all vertebrate species studied.

Metamorphic climax The final and most rapid phase of morphological change when thyroid activity is at its peak.

Paedomorphosis Reproductive maturity is attained while in a larval or branchiate form.

Premetamorphosis Stage of amphibian larval development when the animal grows but little or no morphological change occurs; plasma thyroid hormone concentrations are low.

Prolactin (PRL) Simple polypeptide hormone produced by the anterior pituitary gland that stimulates lactation in mammals and has antimitamorphic effects in amphibians.

Prometamorphosis Stage of amphibian larval development when metamorphosis begins. Hindlimb growth and development is evident externally. The thyroid gland becomes active and secretes thyroid hormone in response to increasing plasma concentrations of pituitary thyroid stimulating hormone (TSH).

Thyroid stimulating hormone (TSH) Glycoprotein hormone comprised of two subunits produced by the anterior pituitary gland that stimulates the production of thyroid hormone by the thyroid gland.

Thyrotropin-releasing hormone (TRH) Tripeptide produced in the hypothalamus and extrahypothalamic sites that stimulates the release of TSH by the anterior pituitary gland.
2.09.1 Most Amphibian Species Have Complex Life Cycles

Amphibians exhibit considerable diversity in behavioral, physiological, and life history strategies. They are geographically widespread, occupying a diverse range of habitats. The life history strategies of amphibian species include complex life cycles (e.g., metamorphosis) and direct development (Callery et al., 2001). The majority of amphibian species exhibit complex life cycles, and thus have two very different life stages that are affected differently by environmental factors. Most urodele amphibians (frog) larvae are aquatic, and tadpoles are found in a wide variety of habitats, ranging from water-filled crevices in rocks, logs, or leaves to larger ponds or streams. Most then undergo morphological, biochemical, and physiological transformation into adults, which are sensitive to different environmental variables than larvae, due to this shift in habitat (Duellman and Trueb, 1994). Some amphibians have lost the larval form and develop directly into the adult morphology (direct development); others do not metamorphose but reproduce in the aquatic habitat while retaining the larval morphology (paedomorphosis).

Metamorphosis is a stage of many amphibian life cycles characterized by dramatic morphological transformation that is accompanied by a transition in ecological niche and behavioral mode. Amphibians that undergo a metamorphosis exhibit strong variation, both between and within species, in the duration of the larval period (Wilbur and Collins, 1973; Werner, 1986). Larvae encounter diverse ecological conditions during development. Variation in abiotic factors (e.g., water availability, temperature, photoperiod) as well as biotic factors (e.g., intra- and interspecific competition, predation) can interact in complex ways to influence larval growth and development (Semlitch, 1987; Sredl and Collins, 1992; Rowe and Dunson, 1995; Taylor and Scott, 1997; Alvarez and Nieve, 2002; Relyea, 2002; Downie et al., 2004; Relyea, 2007). The timing of metamorphosis is a central amphibian life history trait that likely reflects the quality and relative permanence of the larval habitat. Species that breed in predictable habitats (i.e., permanent or semipermanent lakes and ponds) tend to have longer larval periods. Species that breed in unpredictable habitats (i.e., ephemeral pools) generally have much shorter larval periods (Denver, 1997b).

Wilbur and Collins (1973) suggested that there is a threshold of minimum body size that must be reached before metamorphosis is possible and that larval growth rates determine the timing of metamorphosis after this minimum size has been attained. Werner (1986) added mortality risk in the larval and adult habitat to the list of factors that ultimately influence metamorphosis. The effects of environmental factors may differ depending on the animal’s stage of development. These observations led Day and Rowe (2002) to incorporate developmental thresholds into the Wilbur–Collins model. Environmental factors that influence growth rate or mortality risk therefore should alter the timing of metamorphosis, and the effects of the environment may be influenced by the stage of development that has been achieved. For example, the same factor may be inhibitory to growth if present early in the larval phase or stimulatory to development if present during metamorphosis (e.g., population density, food availability, pond drying or predation – reviewed by Denver, 1997b). Thus, body size and stage of development may interact in complex ways to determine the phenotypic response to specific environmental variables.

Amphibian larvae exhibit plasticity in the timing of metamorphosis and can capitalize on favorable conditions for growth as long as such conditions last (up until a genetically determined upper limit to the length of the larval period) (Newman, 1992; Rudolf and Rodel, 2007). Such plasticity may permit amphibian larvae to match their phenotype (morphology, physiology, metamorphic timing) to prevailing environmental conditions. Animals capable of phenotypic plasticity may have a higher probability of surviving in unpredictable habitats compared with those with a genetically fixed, or canalized phenotype (Stearns, 1989; Newman, 1992; Van Buskirk, 2002; Gomez-Mestre and Buchholz, 2006).

The upper and lower limits to the length of the larval period are determined by genetic factors that are subject to natural selection. The plasticity of larval period length within these limits is also subject to natural selection and is influenced at both proximate and ultimate levels by the environment. While metamorphic timing is determined by both genetic and environmental factors, its expression depends on the development and activity of endocrine glands and the actions of the hormones that these glands produce (see below).

Among the most extreme evolutionary modifications of the ancestral, complex life history is paedomorphosis. Most amphibian larvae undergo a metamorphosis to an adult form before becoming sexually mature. Some species of urodele amphibians (e.g., salamanders, newts) exhibit paedomorphosis, where reproductive maturity is attained while in a larval or branchiate form. Paedomorphosis refers to the retention of juvenile characteristics in sexually mature adults (Gould, 1977). Paedomorphosis can either be obligate or facultative depending on the species. Obligate paedomorphs never undergo metamorphosis and remain in an aquatic habitat their entire lives (e.g., Necturus, Proteus, Ambystoma, Ambystoma mexicanum – axolotl). Facultatively paedomorphic species can either become paedomorphic and remain in the aquatic habitat, or metamorphose and move into the terrestrial environment where they become sexually mature (e.g., Ambystoma tigrinum, Ambystoma talpoideum, Ambystoma gracile, Notophthalmus viridescens) (Duellman and Trueb, 1994). The developmental ‘decision’ to become paedomorphic or to metamorphose in facultative species depends on the prevailing environmental conditions rather than the animal’s genotype (Harris, 1987; Semlitch, 1987; Licht, 1992; Jackson and Semlitch, 1993; Denoel and Poncin, 2001) and may be controlled by the interplay of antagonistic hormonal pathways (Wakahara, 1994, 1996; Rosenkilde and Ussing, 1996). The reader is referred to a recent review by Johnson and Voss (2013) for a detailed discussion of the hormonal basis for paedomorphosis in salamanders.

2.09.2 Endocrinology of Metamorphosis

2.09.2.1 Overview

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. Gudernatsch (1912) first showed that the vertebrate thyroid gland contained a factor that could induce precocious metamorphosis if fed to tadpoles. This compound, later identified as 3,5,3',5'-tetraiodothyronine (thyroxine) (Kendall, 1915; Harrington, 1926; Harrington and George, 1927) and referred to as thyroid hormone (TH), is now known to be the primary hormone controlling amphibian metamorphosis. Thyroid hormone is required for amphibian metamorphosis (Brown and Cai, 2007); the hormone initiates gene expression programs in diverse tissues that lead to cell proliferation, death, differentiation, or migration (Brown and Cai, 2007). While hormones produced by the anterior pituitary gland and the interrenal glands (amphibian homologs of the mammalian adrenal cortex; corticosteroids – CS) influence the rate of metamorphosis by controlling TH production and action on target tissues, exogenous TH alone can induce the entire suite of tissue transformations. Furthermore, chemical or surgical thyroidectomy results in metamorphic stasis (Dodd and Dodd, 1976; Kikuyama et al., 1993). 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.

The work of William Etkin laid much of the foundation for our current understanding of the endocrine control of metamorphosis. Etkin, (1968) proposed a model for the hormonal changes that occur during amphibian metamorphosis. He also coined the terms in common use today among amphibian endocrinologists for describing the stages of anuran development: ‘premetamorphosis,’ when the larvae grow but little or no morphological change occurs and plasma TH concentrations are low; ‘prometamorphosis,’ when hindlimb growth accelerates and plasma TH concentration rises; ‘metamorphic climax,’ the final and most rapid phase of morphological change when thyroid activity is at its peak (Dodd and Dodd, 1976; White and Nicoll, 1981; Table 1).

The objective of the following section is to describe the cast of endocrine characters that interact to control metamorphosis.

### Table 1: A comparison of three of the most widely cited staging tables for postembryonic, feeding stages of anuran larvae

<table>
<thead>
<tr>
<th>NF staging for Xenopus laevis</th>
<th>Major, common diagnostic features/morphological changes</th>
<th>TK staging</th>
<th>Gosner staging</th>
<th>Terminology of Etkin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–45</td>
<td>Nonfeeding stages (comparable to Shumway stages 1–24)</td>
<td>I</td>
<td>1–25</td>
<td>Premetamorphosis</td>
</tr>
<tr>
<td>46</td>
<td></td>
<td>II</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>47–48</td>
<td>Feeding begins</td>
<td>III</td>
<td>27</td>
<td></td>
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<tr>
<td>49–50</td>
<td></td>
<td>IV</td>
<td>28</td>
<td></td>
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<tr>
<td>51</td>
<td></td>
<td>V</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>Foot paddle stages</td>
<td>VI</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td></td>
<td>VII</td>
<td>31</td>
<td></td>
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<tr>
<td>54</td>
<td></td>
<td>VIII</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>Hindlimb stages</td>
<td>IX</td>
<td>33</td>
<td></td>
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<tr>
<td>56</td>
<td></td>
<td>X</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>57–58</td>
<td>Tadpole reaches maximum length</td>
<td>XI</td>
<td>35</td>
<td></td>
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<tr>
<td>59</td>
<td></td>
<td>XII</td>
<td>36</td>
<td>Prometamorphosis</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>XIII</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>Rapid tail resorption begins, front limbs erupt*</td>
<td>XIV–XVI</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>62</td>
<td></td>
<td>XV</td>
<td>39–40</td>
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<td>63</td>
<td></td>
<td>XVI</td>
<td>40</td>
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<td>64</td>
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<td>XVII</td>
<td>41</td>
<td></td>
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<tr>
<td>65</td>
<td></td>
<td>XVIII</td>
<td>42</td>
<td>Climax</td>
</tr>
<tr>
<td>66</td>
<td>Stump of tail remains</td>
<td>XIX</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>Tail completely resorbed, juvenile frog</td>
<td>XX</td>
<td>44</td>
<td></td>
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<td></td>
<td></td>
<td>XXI</td>
<td>45</td>
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<td></td>
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<td>XXII</td>
<td>46</td>
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<td>XXV</td>
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</tbody>
</table>

Note: Table is derived from similar tables published by Nieuwkoop and Faber (1956), Dodd and Dodd (1976), and Kikuyama et al. (1993) with the addition of Gosner staging. Note that the table is modified somewhat with respect to the table published by Kikuyama et al. (1993) with deference to the comparison between Xenopus laevis and the staging of Rana pipiens (Taylor and Kollros, 1946) made by Nieuwkoop and Faber (1956). Comparison of Taylor and Kollros (1946) with Gosner (1960) staging tables is based on that of Gosner (1960).

*In Xenopus species the front limbs do not erupt, but instead develop outside of the body cavity.

*Stages of Nieuwkoop and Faber (1956).
*Stages of Taylor and Kollros (1946).
*Stages of Gosner (1960).
*Stages of Shumway (1940).
For each endocrine axis involved in metamorphosis I will first examine its developmental schedule. This should allow predictions of when the endocrine system is sufficiently developed to allow the animal to become competent to respond to the external environment. I will also examine the multiple levels at which the activity and functioning of each endocrine axis can be regulated. The goal, which is addressed in Section 2.09.3 on integration, is to understand how the endocrine system determines the timing of metamorphosis and mediates environmental effects on amphibian development.

2.09.2.2 Thyroid Hormone

2.09.2.2.1 Role of Thyroid Hormone in Amphibian Development

Perhaps the most striking characteristic of amphibian metamorphosis, from the perspective of hormonal control, is that a single signaling molecule, produced by a highly restricted group of cells (the thyroid epithelial cells), can orchestrate the entire suite of molecular, biochemical, and morphological changes. Depending on the tissue, TH can induce cell proliferation, cell death, differentiation, or migration. Target cells for TH are now known to activate both similar and different sets of genes according to the concentration of this single signaling molecule. Specific tissues exhibit different dose sensitivities to TH, and the challenge for investigators studying the molecular basis of TH action during metamorphosis is to determine how and why individual tissues respond differently to the hormone and exhibit differential dose responses (see Brown and Cai, 2007). Thyroid hormone regulation of metamorphosis has been best studied in anuran amphibians, which is the primary focus of this chapter, but it is important to note that TH is also the primary morphogen in metamorphosing urodele (salamander) species (reviewed by Johnson and Voss, 2013).

2.09.2.2.2 Thyroid Gland Development and Hormone Production

The thyroid gland develops early in the amphibian embryo when the anlage consists of a thickening of the pharyngeal epithelium; these cells are capable of synthesizing small iodo-proteins (reviewed by Dodd and Dodd, 1976; Regard et al., 1978). The gland matures functionally at the time of hatching when it separates into two distinct lobes and is essentially completely developed by late premetamorphosis/early prometamorphosis (Nieuwkoop and Faber, 1956; Saxen et al., 1957a,b; Kaye, 1959, 1961; Dodd and Dodd, 1976; Regard et al., 1978). Multiple measures of thyroid activity including radioiodine uptake, gland ultrastructure, and plasma concentration or tissue content of THs show that thyroid activity increases markedly during prometamorphosis (Table 1; Figure 1), peaks at metamorphic climax, and declines thereafter to reach an ‘adult’ level of activity (Kaye, 1959, 1960; Dodd and Dodd, 1976; Regard et al., 1978; Kikuyama et al., 1993). Ultrastructural analyses show a dramatic increase in thyroid follicular cell height during prometamorphosis with a peak at metamorphic climax that corresponds to the peak in plasma concentrations (and tissue content) of THs (Dodd and Dodd, 1976; Regard et al., 1978).

When Etkin proposed his endocrine-based model for metamorphosis, investigators at the time did not have sensitive and quantitative methods for determining plasma TH concentrations. Early methods relied on the determination of protein-bound iodide to estimate plasma TH titers (Just, 1972). Subsequently, sensitive and specific radioimmunoassays (RIAs) were developed that allowed determinations of plasma thyroxine (T₄; the primary product of the thyroid gland) and 3,5,3’-triiodothyronine (T₃; derived from T₄ by monodeiodination in target tissues) concentrations during metamorphosis. These studies confirmed earlier studies and the predictions of Etkin by demonstrating low-to-nondetectable plasma TH concentrations during prometamorphosis, increasing concentrations during metamorphosis, and a dramatic peak at metamorphic climax (Figure 1(b); Leloup and Buscaglia, 1977; Miyauchi et al., 1977; Regard et al., 1978; Mondou and Kaltenbach, 1979; Suzuki and Suzuki, 1981; Weil, 1986; Niinuma et al., 1991a; Weber et al., 1994; Denver, 1998a; Krain and Denver, 2004).

Because of the difficulty of obtaining blood from small tadpoles for analysis by RIA, until recently only those species with tadpoles large enough to obtain a serum sample were analyzed. Thus, most blood measurements have been done on ranid species (e.g., Rana catesbeiana; Rana clamitans); however, Leloup and Buscaglia (1977) and Tata et al. (1993) measured THs in plasma pools of Xenopus laevis; see also Buscaglia et al. (1985) for measures of plasma T₃ and T₄ in other Xenopus spp.). In species with small tadpoles, developmental changes in TH content of whole bodies and individual tissues have been determined. These analyses have shown that changes in whole body TH content in the smaller species essentially parallel changes observed in the plasma of tadpoles of the larger species (Bufo japonicus (Niinuma et al., 1991a); Spea hammondii (Denver, 1993, 1998a); X. laevis (Krain and Denver, 2004); Bufo marinus (Weber et al., 1994)). The peak in whole-body T₃ and T₄ coincides with peak uptake of ¹³¹I in B. japonicus (Niinuma et al., 1991a). Thus, it is likely that determination of whole-body hormone content provides a reasonable estimate of physiological changes in TH production in species for which blood samples are unobtainable.

2.09.2.2.3 Thyroid Hormone Metabolism

The major product of the amphibian thyroid gland is T₄ with minor amounts of T₃ produced (Rosenkilde, 1978; Buscaglia et al., 1985). The result is that plasma T₄ concentration tends to be an order of magnitude greater than T₃ (Regard et al., 1978; Larras-Regard et al., 1981). The only case where this relationship may not hold is for X. laevis where the reported plasma concentrations of T₃ and T₄ are very similar, and the T₃/T₄ ratio may even exceed one at metamorphic climax (Leloup and Buscaglia, 1977; Buscaglia, 1985). Measures of tissue content of T₄ and T₃ in various species show that the two hormones are present in roughly similar amounts (Niinuma et al., 1991a; Weber et al., 1994; Denver, 1997a, 1998a). Although a comprehensive analysis of both blood concentrations and tissue contents of THs has not been done for any species, it is likely that the higher T₃:T₄ ratio in tissues compared with plasma reflects high tissue 5’-monodeiodinase activity (see below).

An important point of control of TH bioactivity is at the target tissues, where monodeiodinase enzymes convert T₄ to T₃, or inactivate T₄ and T₃ (Figure 2). The monodeiodinases
catalyze two basic reactions: a 5' monodeiodination (outer ring) that results in bioactivation and a 5 monodeiodination (inner ring) that results in bioinactivation of the substrate, T4 or T3. 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', type II 5', and type III 5 deiodination (St Germain et al., 2009). Types II and 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 et al., 1997; Dubois et al., 2006; Kuiper et al., 2006). An iodotyrosine deiodinase that is involved with recycling of iodine from mono- and diiodotyrosine is expressed in the thyroid gland and is strongly induced by TH in the intestine during metamorphosis via a TH response element (TRE) located in the promoter of the gene (Fujimoto et al., 2012).

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; Cai and Brown, 2004; Brown, 2005; Duarte-Guterman et al., 2012). The expression patterns correlate with the asynchronous tissue morphogenesis and the roles that the deiodinases play in modulating intracellular T3 concentration during metamorphosis (Brown, 2005; St Germain et al., 2009). In many cells
Figure 2  Central and peripheral organization of the thyroid and stress endocrine axes controlling amphibian metamorphosis. Shown is a schematic representation of the hypothalamo–pituitary–thyroid (HPT) and hypothalamo–pituitary–interrenal (HP; 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 CRH 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, thyroxine binding globulin, and albumin). ACTH acts on interrenal cortical cells in the interrenal glands to stimulate biosynthesis and 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). Thyroid hormone receptors (TR) form heterodimers with retinoid X receptors (RXR) and are bound to DNA in the unliganded form where they actively repress gene transcription. Upon thyroid hormone
both enzymes may be expressed, and the relative expression levels may establish a type of push–pull mechanism that regulates intracellular T3 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 et al., 1998); both Dio3 mRNA and 5’ deiodinase activity increase during late prometamorphosis (Nieuwkoop-Faber (NF) stages 59–61; refer Table 1 for a definition of developmental stages) but then decline sharply at metamorphic climax (St Germain et al., 1994; Brown et al., 1996). 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 fibroblasts, is undetectable before late prometamorphosis at which time expression increases markedly and is maintained through the end of metamorphosis (Cai and 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 et al., 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 monodeiodinase 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 such as 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 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 et al., 2007). Thyroid hormone induces cell proliferation in the early prometamorphic tadpole brain, but cells of the neurogenic zone become refractory to TH action on cell proliferation as the animals approach metamorphic climax (Denver et al., 2009). The decline in cell proliferation is likely due to processes, probably 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 (Huang et al., 2001; Cai and Brown, 2004; Manzon and Denver, 2004; discussed more below). Physiological roles for tissue monodeiodinases in the timing of metamorphosis are supported by experiments with iopanoic acid and transgenesis overexpression of Dio3 (Buscaglia, 1985; Galton, 1989; Becker et al., 1997; Huang et al., 1999, 2001; Marsh-Armstrong et al., 1999; Cai and Brown, 2004).

2.09.2.4 Thyroid Hormone Transport in Blood

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 (Figure 2). 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). Trans-thyretin (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). However, the situation in amphibia is the reverse, where TTR binds T₃ with greater affinity than T₄ (Yamauchi et al., 1993, 1998, 1999, 2000). 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 protein is present in tadpoles during metamorphosis and metamorphosis when thyroid activity is increasing, but declines at metamorphic climax (Yamauchi et al., 1998, 2000; Prapunpoe et al., 2000). The free hormone hypothesis (Mendel, 1989; Ekins, 1990) leads to the prediction that TTR during pre- and early metamorphosis 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 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 increase at metamorphic climax, but so 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 metamorphosis compared with premetamorphosis or metamorphic climax. Furthermore, given the lower affinity of TTR for T₄ compared with T₃, one would predict that the clearance rate for T₄ would be higher than that of T₃.

### 2.09.2.2.6 Mechanisms of Thyroid Hormone Action

Tadpoles become competent to respond to exogenous TH at the time of hatching (Tata, 1968). This establishment of
competence to respond to the hormone likely depends on the expression of TRs (Shi et al., 1996). TRs are ligand-activated transcription factors that belong to the steroid hormone receptor superfamily (Mangelsdorf et al., 1995). There are two TR genes, termed α and β, in all vertebrates studied to date; owing to its pseudotetraploidy, X. laevis possesses four TR genes, two αs and two βs (Buchholz et al., 2006). The two X. laevis TRα genes each appear to give rise to single, unique proteins; whereas, alternative mRNA splicing of TRβ transcripts can give rise to two different receptor isoforms for each TRβ gene (Buchholz et al., 2006).

The TRβ genes are first expressed shortly after hatching in X. laevis, and their expression rises during premetamorphosis and remains high throughout metamorphosis (Baker and Tata, 1990; Yaita and Brown, 1990; Banker et al., 1991; Kawahara et al., 1991). It has been hypothesized that the early expression of TRβ establishes hormone responsiveness of tadpole tissues (Baker and Tata, 1990; Shi et al., 1996). TRβ mRNA is not detected until early metamorphosis, but its expression increases during metamorphosis in parallel with TH synthesis (Yaita and Brown, 1990; Kawahara et al., 1991; Baker and Tata, 1992; Kanamori and Brown, 1992). Several studies have shown that the TR genes are upregulated by T3 in X. laevis and R. catesbeiana (Yaita and Brown, 1990; Kawahara et al., 1991; Schneider and Galton, 1991; Helbing et al., 1992) (a phenomenon termed ‘autoinduction’; see Tata et al., 1993). A thyroid hormone response element to which TRs can bind and regulate transcription has been identified in the X. laevis TRβA gene (Ranjan et al., 1994; Machuca et al., 1995). Autoinduction may require the upregulation of accessory transcription factors such as the immediate early, TH-inducible gene Krüppel-like factor 9 (Klf9; aka basic transcription element binding protein 1; BTEB1) (Bagamasbad et al., 2015; Hu et al., 2016).

A central role for TRs in metamorphosis is supported by transgenic studies in X. laevis. For example, transgenic expression of a dominant negative TR blocks metamorphosis (Schreiber et al., 2001; Buchholz et al., 2003), while expression of a dominant positive TR promotes metamorphic changes in the absence of TH (Buchholz et al., 2004). Aran et al. (2014) recently showed that autoinduction of TR α and β and the actions of exogenous T3 on epibranchial remodeling were strongly reduced or eliminated in paedomorphic versus metamorphic populations of the Oklahoma salamander Eurycea typhles. These findings provide further support for the essential role of TH and TRs in metamorphosis, and their roles in the expression of alternative developmental modes in amphibians.

Specific functions for the different receptor subtypes in amphibians are poorly understood. Expression studies suggest differential roles, as do pharmacological studies with TR subtype-selective agonists. For example, studies with the TRα-selective agonist CO23 support that this receptor subtype is involved in cell proliferation (Ocasio and Scanlan, 2006; Denver et al., 2009), while studies with the TRβ-selective agonists GC1 and GC24 support that this subtype is primarily involved with tissue resorption (apoptosis) and cell differentiation (Furlow et al., 2004; Ocasio and Scanlan, 2006; Denver et al., 2009). Experimental studies addressing specific functions for the different TRs in amphibians were limited by the inability to delete, or knockdown, the TRs. Recent technological developments using transcription activator-like effector nucleases (TALENs) for genome editing have shed light on the role of TRz in tadpole development and metamorphosis. Two groups recently used TALENs to introduce mutations into the TRz locus in Xenopus tropicalis (Choi et al., 2015b; Wen and Shi, 2015). Mutations in TRz led to enhanced growth of premetamorphic tadpoles and the acceleration of the onset of metamorphosis (Wen and Shi, 2015). Although the timing of the initiation of metamorphosis was accelerated, these mutant tadpoles were resistant to exogenous T3 and exhibited a delay in spontaneous metamorphosis. Gene expression analyses showed increased baseline mRNA levels, but the loss of T3 response in TR target genes (Choi et al., 2015b; Wen and Shi, 2015). Together, these findings support that the aporeceptor normally represses metamorphic genes in larvae, and when removed shortens the larval period. However, liganded TRz is required for establishing tissue responsiveness to the hormone during metamorphosis, and so these studies provide critical support for the dual function model for TR actions during metamorphosis (Buchholz et al., 2006; Shi et al., 2012; Grimaldi et al., 2013a). Future studies using TALENS and CRISPR/Cas9 genome editing should shed further light on the different roles of the TR subtypes in tadpole development.

Thyroid hormone receptors function as dimers; that is, the DNA consensus sequences that TRs bind to are six nucleotides in length and are referred to as ‘half sites.’ Two of these half sites comprise a TRE (Williams and Brent, 1995). These TREs can be located within the promoter, within the structural part of the gene, or upstream of the transcription start site. Homodimers of TRα or TRβ can form on most TREs, but the preferred configuration appears to be as a heterodimer with retinoid X receptor (RXR) (Wong and Shi, 1995; Puzianowska-Kuznicka et al., 1997). TR-RXR heterodimers bind DNA and transactivate TRE-containing genes much more effectively than TR homodimers. In the unliganded form, the TR-RXR complex functions as a transcriptional repressor (Wong and Shi, 1995). The TR-RXR heterocomplex recruits cofactor proteins that mediate the repressive or activation actions of the complex (Shi, 2000b; McKenna and O’Malley, 2002). The unliganded receptor may have important developmental functions in the premetamorphic tadpole (discussed below). The TR and RXR genes exhibit more or less coordinate regulation during metamorphosis, and this coordination may be essential to the timing of tissue-specific changes (Wong and Shi, 1995). Current studies are focused on identifying TREs throughout the genome in different tadpole tissues using chromatin immunoprecipitation (ChIP) combined with high-throughput sequencing (Grimaldi et al., 2013b).

Hormone binding to the TR-RXR receptor complex induces gene expression in target tissues. The TRs cause modifications of chromatin through recruitment of coactivator and corepressor proteins (Shi et al., 2012; Grimaldi et al., 2013a). In the unliganded state, TRs function primarily as transcriptional repressors, recruiting corepressors such as NCoR and SMRT which then recruit histone deacetylases (Tomita et al., 2004). This leads to a compact, repressive chromatin structure (Sachs et al., 2001; Jones and Shi, 2003). When TH binds to TRs, corepressors are exchanged for
coactivators, leading to transcriptional derepression and transactivation. Many of the coactivators have intrinsic histone acetyl transferase activity, leading to the addition of acetyl groups to lysine residues on histone tails. Coactivator expression and recruitment to target genes is correlated with tissue transformation and gene activation (Paul and Shi, 2003; Paul et al., 2005b), and transgenic analysis suggests that the recruitment of coactivators by TRs is essential for metamorphosis to proceed (Paul et al., 2005a, 2007). A role for unliganded TR in tadpole development, whereby it represses the expression of adult genes (Sachs et al., 2002; Sato et al., 2007), has led to the development of the dual function model for TR action during metamorphosis. This model proposes that unliganded TRs (the aporeceptors) play an important role in development by repressing adult genes in the tadpole prior to the onset of thyroid activity, but activate expression of metamorphosis-associated genes when ligand is present (reviewed by Buchholz et al., 2006; Grimaldi et al., 2013a; Shi, 2013). The aporeceptors have also been implicated in heart development and the maturation of the brain and other organs prior to the onset of thyroid gland function in mammals (Bernal and Morte, 2013). Discussion of the characteristics of the gene regulation cascades and the functions of the gene products induced by TH in different tissues during metamorphosis is beyond the scope of this chapter. For detailed discussion of these topics the reader is referred to the following reviews: Furlow and Neff (2006), Buchholz et al. (2006), Brown and Cai (2007), and Grimaldi et al. (2013a).

2.09.2.3 Corticosteroids

In addition to TH, corticosteroids (CS), 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 et al., 1961; Macchi and 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 (Jaffe, 1981; Krug et al., 1983; Jolivet-Jaudet and Leloup-Hatey, 1984; Kikuyama et al., 1986; Carr and Norris, 1988; Niinuma et al., 1989; Klosa et al., 1997; Denver, 1998a; Glennemeier and Denver, 2002a; Krain and Denver, 2004). The majority of these studies showed low-to-nondetectable corticosteroids during premetamorphosis, and a marked increase at metamorphic climax, more or less in parallel with the rise in THs. The only exception to this rule is whole-body corticosteroid content in X. laevis. Klosa et al. (1997) reported that whole-body corticosterone content in X. laevis increases during premetamorphosis to reach a peak at NK stage 48 and then declines during prometamorphosis and is low at metamorphic climax. Klosa et al. (1997) also measured whole-body aldosterone and found a similar increase during premetamorphosis, but the peak production was during early prometamorphosis (NK stage 54) and it declined thereafter. Glennemeier and Denver (2002a) obtained similar results with corticosterone, although in X. laevis there was a small increase at metamorphic climax. Whether these findings in X. laevis represent species differences or whether changes in whole-body corticosteroid content are not representative of changes in plasma concentrations is currently unknown.

Corticosteroids, being lipophilic, are transported in blood bound to plasma proteins. Corticosteroid-binding globulin (CBG) is the primary plasma protein to which corticosteroids bind in mammals, although albumin also plays a transport role (Hammond, 1990; Rosner, 1990). Recently, binding properties of a putative CBG present in amphibian serum (A. tigrinum) were reported by Orchinik et al. (2000). However, the expression of CBG has not been studied in amphibians.

2.09.2.3.1 Development of and Hormones Produced by Amphibian Interrenal Glands

The interrenal gland is generally less active in early, premetamorphic developmental stages and more active during prometamorphosis and metamorphic climax (Dodd and Dodd, 1976). The ultrastructural appearance of X. laevis interrenal cells indicates relative inactivity in mid-prometamorphs, increasing to peak activity at metamorphic climax (reviewed by Dodd and Dodd, 1976; however, see below for contrary evidence). Activity of the interrenal enzyme, Δ4-3β-hydroxysteroid dehydrogenase (HSD) is present throughout development in R. catesbeiana and X. laevis but increases at metamorphic climax in R. catesbeiana (Hsu et al., 1980; Kang et al., 1995). Carr and Norris (1988) found a similar pattern for plasma corticosterone and interrenal HSD activity in the tiger salamander, A. tigrinum.

Radioimmunoassays for corticosteroids have been done on plasma samples collected throughout the metamorphic period for several amphibian species: R. catesbeiana (Jaffe, 1981; Krug et al., 1983; Kikuyama et al., 1986); B. japonicas (Niinuma et al., 1989); X. laevis (Jolivet-Jaudet and Leloup-Hatey, 1984); A. tigrinum (Carr and Norris, 1988). Whole-body measures of corticosteroid content have also been determined throughout development: S. hammondii (Denver, 1998a); X. laevis (Klosa et al., 1997; Krain and Denver, 2004); Rana pipiens (Glennemeier and Denver, 2002a). The majority of these studies showed low-to-nondetectable corticosteroids during premetamorphosis, and a marked increase at metamorphic climax, more or less in parallel with the rise in THs. The only exception to this rule is whole-body corticosteroid content in X. laevis. Klosa et al. (1997) reported that whole-body corticosterone content in X. laevis increases during premetamorphosis to reach a peak at NK stage 48 and then declines during prometamorphosis and is low at metamorphic climax. Klosa et al. (1997) also measured whole-body aldosterone and found a similar increase during premetamorphosis, but the peak production was during early prometamorphosis (NK stage 54) and it declined thereafter. Glennemeier and Denver (2002a) obtained similar results with corticosterone, although in X. laevis there was a small increase at metamorphic climax. Whether these findings in X. laevis represent species differences or whether changes in whole-body corticosteroid content are not representative of changes in plasma concentrations is currently unknown.

Corticosteroids exert complex effects on tadpole growth and development. Depending on the animal’s developmental stage
and TH status, CS can accelerate or decelerate metamorphosis (Kulkarni and Buchholz, 2014). If elevated during premetamorphosis, CS typically inhibit tadpole growth and slow development (Frieden and Naile, 1955; Kobayashi, 1958; Gray and Janssens, 1990; Hayes et al., 1993; Wright et al., 1994; Hayes, 1995; Darras et al., 2002; Glennemeier and Denver, 2002b; Belden et al., 2005; Hu et al., 2008).

However, CS have been found to accelerate TH-induced and spontaneous metamorphosis (Frieden and Naile, 1955; Kikuyama et al., 1983, 1993; Gray and Janssens, 1990; Wright et al., 1994; Hayes, 1995; Darras et al., 2002; Kuhn et al., 2004, 2005). 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 metamorphosis accelerate metamorphosis. The mechanisms of CS inhibition of growth in tadpoles have not been investigated, but based on studies in mammals these actions could be manifest at multiple levels that likely include diverse catabolic actions (Sapolsky et al., 2000) and perhaps decreased anterior pituitary growth hormone (GH) biosynthesis (Harvey et al., 1995).

Corticosteroids, such as all steroid hormones, act primarily through binding to receptors that function as ligand-dependent transcription factors. These receptors are members of the same superfamily of receptor proteins that include the TH receptors (see above). Corticosteroid receptors are found primarily in the cytosol in the absence of ligand where they are complexed with a series of heat shock proteins and immunophilins (a foldosome) that serve to maintain the receptors in a conformation that favors ligand binding (Pratt and Toft, 1997). Binding of hormone results in dissociation of the foldosome complex and translocation of the receptor to the nucleus (Pratt and Toft, 1997). Vertebrates possess two distinct corticosteroid receptors that were originally identified in mammals based on their differential binding affinities: the high-affinity type I receptor (also called the mineralocorticoid receptor; MR) and the lower-affinity type II receptor (also called the glucocorticoid receptor; GR). The GR and MR belong to the nuclear hormone receptor superfamily, and phylogenetic analysis suggests that these two receptors arose by a gene duplication event in the gnathostome lineage (Thornton, 2001; Bridgham et al., 2006). Homologous genes to mammalian receptors have been isolated in X. laevis (Gao et al., 1994a,b; Csikos et al., 1995). The distribution in the brain and the regulation of expression by corticosteroids of the GR were recently reported in X. laevis (Yao et al., 2008).

The molecular mechanisms by which corticosteroids promote metamorphosis involve, at least in part, the enhancement of TH bioactivity through increased TR and monodeiodinase expression. Corticosteroids were shown to increase maximal nuclear binding capacity for T3 in a dose-dependent manner (Niki et al., 1981; Suzuki and Kikuyama, 1983; Kikuyama et al., 1993), 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). The upregulation of TRs enhances cellular responses to TH; for example, forced expression of TRz in Xenopus tadpole tail promoted T3-dependent cell death (Hollar et al., 2011; Nakajima et al., 2012), forced expression of TRβ in XTC-2 cells and tadpole brain enhanced the gene expression response to T3 (Hu et al., 2016), and higher TRz expression was associated with shorter larval periods in spadefoot toads (Hollar et al., 2011). Corticosterone also increased 5’-deiodinase activity and Dio2 mRNA in tadpoles, thereby increasing T3 in target tissues (Galton, 1990; Darras et al., 2002; Kuhn et al., 2005; Bonett et al., 2010). Notably, the action of CORT on Dio2 was synergistic with T3 in tadpole tail (Bonett et al., 2010). Darras et al. (2002) showed that treatment with dexamethasone increased hepatic D3 and brain D2 activities, plasma T3, and induced metamorphic changes (without concomitant TH treatment) in the axolotl. Kuhn et al. (2005) showed that treatment with CRH plus T4 caused a strong synergistic activation of brain D2 activity in the axolotl.

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, Klf9, a direct T3 target gene, is induced by CORT (Bonett et al., 2009) and is superinduced in tadpole tissues with rapid kinetics by combined treatment with T3 plus CORT (Bagamasbad et al., 2015). Similar synergistic regulation of Klf9 by T3 and corticosteroids was seen in mouse brain and neuronal cells, and the synergy was found to be mediated by an ultrasonically conserved nuclear receptor enhancer module (Bagamasbad et al., 2015). There may be other genes that are synergistically regulated by TH and CS that could explain the mechanism by which these two hormones cooperate to accelerate metamorphosis (see Kulkarni and Buchholz, 2012, 2014). These findings suggest that synergistic gene regulation by TH and corticosteroids may be a general and important phenomenon in animal development.

In summary, the dose of corticosteroid administered, the stage at which the hormone is given, and whether it is administered with TH determine the developmental effects of the steroid. Whether these effects represent physiological actions remains to be determined. If these actions turn out to be physiologically relevant, then one would predict that increased corticosteroid biosynthesis (perhaps in response to a stressor) in premetamorphic tadpoles might retard growth and delay metamorphosis. Conversely, increased corticosteroids in metamorphic tadpoles might retard growth but accelerate metamorphosis.

### 2.09.2.4 Neuroendocrine Control of Metamorphosis

The vertebrate neuroendocrine system is comprised of the hypothalamus and the pituitary gland. The major pituitary hormones and their roles in amphibian development are described above. The secretion of these pituitary hormones and thus the production of hormones by peripheral endocrine glands (e.g., thyroid and interrenals) are controlled by hypothalamic neurohormones. These neurohormones, termed releasing and release-inhibiting factors, are released from modified nerve terminals in the median eminence into capillaries that drain into the hypophysial portal vessels that deliver blood to the anterior pituitary gland. The importance of hypothalamic control of metamorphosis has long been recognized (reviewed by Denver, 1996). Early studies suggested...
that the pituitary hormones TSH and ACTH are primarily under stimulatory hypothalamic control in amphibians (reviewed by Denver, 1996). The anterior pituitary gland controls both the thyroid gland and the interrenal by production of TSH and ACTH, respectively.

While environmental influences on the timing of metamorphosis can occur at the level of peripheral tissues (e.g., direct thermal effects, osmotic effects), much environmental information is gathered by neural sensory systems and integrated within the hypothalamus to alter the secretion of pituitary hormones and consequently the activity of peripheral endocrine glands. The neuroendocrine system serves as an interface between the central nervous system and the endocrine system and transduces signals obtained through a variety of sensory inputs into appropriate physiological responses (Figure 2).

2.09.2.4.1 Developmental Expression and Regulation of TSH

The increase in thyroid gland growth and biosynthetic activity during metamorphosis is dependent upon the pituitary hormone thyrotropin (thyroid stimulating hormone; TSH). Hypophysectomy of tadpoles arrests the development of the thyroid gland and leads to metamorphic stasis that is reversed during prometamorphosis (Regard and Mauchamp, 1971, 1973; Dodd and Dodd, 1976). 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 (Kaye, 1961; Dodd and Dodd, 1976; Buckbinder and Brown, 1993; Denver, 1996; Manzon and Denver, 2004; Okada et al., 2004, 2009; Korte et al., 2011).

It is likely that the early development of the thyroid gland does not depend on TSH since its development occurs before immunoreactive TSH cells are present in the anterior pituitary (which occurs at NF stage 42 in Xenopus laevis and at similar stages in ranid frogs) (Moriceau-Hay et al., 1982; Tanaka et al., 1991; Gracia-Navarro et al., 1992). However, it cannot be ruled out that small amounts of TSH sufficient to support thyroid development are produced earlier than these stages but cannot be detected due to limitations in the sensitivity of the immunohistochemical detection methods.

While functional thyroid follicles are present at stages that precede the metamorphic rise in TH production, the rate of hormone synthesis is coordinate with the development of the pituitary gland and the production of TSH (Kaye, 1961; Dodd and Dodd, 1976; Buckbinder and Brown, 1993; Denver, 1996; Manzon and Denver, 2004). The amphibian thyroid gland develops sensitivity to TSH during late embroyogenesis (just prior to hatching) as demonstrated by increased radioiodine uptake by thyroids following TSH injection (Kaye, 1961). Immunoreactive TSH was detected in the circulation by RIA in adult and larval callfrogs (Okada et al., 2004), but there have been no measurements of changes in plasma TSH by RIA during metamorphosis. Evidence for an increase in circulating TSH at the early limb bud stage (Taylor Kollros stage III; refer Table 1 for a definition of developmental stages) in R. pipiens tadpoles was provided by Kaye (1961) using indirect measures of $[^{131}I]$ uptake.

Thyrotropin is comprised of two subunits, α and β, that are derived from two separate genes. The α subunit (alpha glycoprotein hormone subunit; α-GSU) is common among the glycoprotein hormones (i.e., the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) and TSH; whereas, the β subunit confers hormonal specificity on the molecule (Pierce and Parsons, 1981).

In X. laevis pituitary expression of TSHβ mRNA and plasma TSH concentration increase throughout metamorphosis (Buckbinder and Brown, 1993; Okada et al., 2000; Manzon and Denver, 2004). Pituitary TSHβ mRNA levels rise from pre-metamorphosis to peak values during late metamorphosis/metamorphic climax (Buckbinder and Brown, 1993; Okada et al., 2000; Manzon and Denver, 2004). 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.

2.09.2.4.2 Neurohormones Regulating TSH

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 TRH gene is expressed in the brain and pituitary of amphibians (Denver and Licht, 1989; Norris and Dent, 1989; Kikuyama et al., 1993; Denver, 1996; Manzon and Denver, 2004; 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 hormone (CRH) and related peptides (e.g., sauvagine, urocortins) (Denver, 2009b,c). CRH-like peptides are well known for their roles in regulating neuroendocrine, autonomic, and behavioral responses to physical and emotional stress in vertebrates (Aguilera, 1998; Yao and Denver, 2007).

2.09.2.4.2.1 Thyrotropin-Releasing Hormone

The tripeptide pyro-glutamyl-histidyl-proline-amide was the first hypothalamic peptide isolated and its structure was determined (Reichlin, 1989). It was named TRH for its ability to stimulate the release of TSH in mammals where it appears to be the principle stimulus of TSH secretion (Morley, 1981). However, its role as a TSH-releasing factor (TRF) in nonmammalian vertebrates is less certain. While TRH is expressed in the brain of larval and adult amphibia, injections of TRH are without effect on the thyroid axis or in altering the timing of tadpole metamorphosis (Norris and Dent, 1989; Kikuyama et al., 1993; Denver, 1996). This is explained by the lack of effect of TRH on the release of TSH by the tadpole pituitary gland (Denver and Licht, 1989; Okada et al., 2004). By contrast, TRH can elevate plasma TH concentrations when injected into adult frogs (Darras and Kuhn, 1982) and can stimulate the release of TSH by adult frog pituitaries in vitro (Denver 1988; Jacobs and Kuhn 1992; Okada et al., 2004). However, the magnitude of the TSH response to TRH in vitro is far lower than that achieved with similar doses of CRH (Denver, 1988; Okada et al., 2004). These findings suggest that pituitary TSH cell responsiveness to TRH is regulated in a developmental stage-specific manner. Expression of the type 2 TRH receptor comes up during late metamorphosis in the X. laevis.
tadpole pituitary; whereas, the type 1 TRH receptor is expressed during pre- and prometamorphosis and is downregulated during metamorphic climax (Manzon and Denver, 2004). In chicken and mammals the type 1 TRH receptor is expressed in thyrotropes and somatotropes (Yu et al., 1998; De Groef et al., 2003a), and TRH receptors are negatively regulated by TH (Hinkle and Goh, 1982; Harvey and Baidwan, 1990; Schomburg and Bauer, 1995). This downregulation of the receptor during metamorphosis, perhaps in response to rising plasma TH concentrations, and perhaps leading to decreased thyrotrope responsiveness to TRH, could account for the failure of this neuropeptide to stimulate TSH release in the tadpole. The type 2 TRH receptor does not appear to be expressed in the rodent pituitary gland (Cao et al., 1998). At present, the possibility that TRH plays a hypophysiotropic role in larval amphibians is uncertain. It could regulate PRL, perhaps via the type 2 TRH receptor, both of which increase at metamorphic climax (Buckhinder and Brown, 1993; Kikuyama et al., 1993; Manzon and Denver, 2004). Further research is needed to clarify the role of TRH in the tadpole.

2.09.2.4.2.2 CRH and Related Peptides

2.09.2.4.2.2.1 CRH-like Peptides

CRH is a 41-amino-acid polypeptide named for its role in inducing release of pituitary ACTH in mammals, a role that is shared in amphibia (Vale et al., 1997; Vale et al., 1981; Turnbull and Rivier, 1997). Members of the CRH family of peptides play central roles in the regulation of neuroendocrine, autonomic, and behavioral responses to physical and emotional stress (Aguilera, 1998; Yao and Denver, 2007). CRH is a member of a family of related peptides in vertebrates that includes the fish urotensins-I, frog sauvagine, and the urocortin peptides (urocortins 1–3) (Dautzenberg and Hauger, 2002; Boorse et al., 2005; Boorse and Denver, 2006). Phylogenetic analysis has shown that tetrapod vertebrates possess four paralogous lineages of CRH-like peptides that likely arose prior to the divergence of the actinopterygian and sarcopterygian fishes (Lovejoy and Balment, 1999; Boorse et al., 2005). These vertebrate peptides share an evolutionary relationship with diuretic peptides present in invertebrates, thus emphasizing the phylogenetically ancient origin of these important hormonal signaling molecules (reviewed by Lovejoy and Balment, 1999). The regulation of ACTH secretion by CRH in mammals is considered to be its primary hypophysiotropic role (Vale et al., 1997).

2.09.2.4.2.2.2 CRH Is a TRF

Shortly after its discovery in 1981, CRH was found to be a potent stimulator of the thyroid axis in larval amphibians and other nonmammalian vertebrates (Denver, 1999, 2009b,c; De Groef et al., 2006). Treatment of amphibian pituitary explants or primary pituitary cells with CRH-like peptides stimulated TSH release (De Groef et al., 2006; Denver, 2009b,c; Okada et al., 2009), and injections of CRH-like peptides elevated whole-body TH content in tadpoles of several species (Gancedo et al., 1992; Denver, 1993, 1997a; Boorse and Denver, 2004). 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 coinubcation with the CRH receptor antagonist α-helical CRH(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 CRH-like peptides. They also suggest that other factors could be involved in the regulation of TSH, or that α-helical CRH(9–41) may have only partial antagonist activity in amphibia as has been found in mammals (Rivier et al., 1984).

Taken together, the findings point to a central, and perhaps primitive role for CRH-like peptides in the regulation of both the thyroid and the interrenal (adrenal) axes.

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

2.09.2.4.2.2.3 Modulation of CRH Actions—Receptors and Binding Protein

CRH actions are mediated by two, G protein-coupled receptors (CRH1 and CRH2) (Dautzenberg and Hauger, 2002) and are modulated by a secreted binding protein (CRH-BP) (Seasholtz et al., 2002). The action of CRH-like peptides on TSH release in the tadpole is mediated by the CRH2 receptor expressed on thyrotropes (Okada et al., 2007a, 2009); whereas, ACTH release may be controlled by the CRH1 receptor in amphibians as it is in mammals (Tonon et al., 1986; Van Pett et al., 2000; De Groef et al., 2003a,b; Okada et al., 2009). In X. laevis CRH1 receptor mRNA was expressed during premetamorphosis, and its level increased during prometamorphosis, reaching a plateau through metamorphic climax (Manzon and Denver, 2004). In contrast, mRNA for the CRH2 receptor was very low during pre- and early metamorphosis, but increased dramatically during late metamorphosis and metamorphic climax. The expression of the CRH2 receptor in the tadpole pituitary paralleled the increase in sensitivity of the pituitary to CRH-like peptides during metamorphosis (Manzon and Denver, 2004). These findings support the hypothesis that the competence of tadpole pituitary thyrotropes to respond to hypothalamic CRH depends on the upregulation of the CRH2 receptor during late metamorphosis.

2.09.2.4.3 Other Neurohormones That Regulate TSH Secretion

According to studies from Sakae Kikuyama’s laboratory, ~50% of the TSH releasing activity of hypothalamic extracts (derived
from both tadpoles and adult frogs) can be blocked by coincubation of adult bullfrog pituitary cells with 10−4 M CRH receptor antagonist α-helical CRH(9-41) (Ito et al., 2004). These findings suggest that a significant proportion of TSH releasing activity in the amphibian hypothalamus is contributed by CRH-like peptides. They also suggest that other factors may be involved in the regulation of TSH or that α-helical CRH(9-41) may not fully antagonize CRH-like peptide activity in hypothalamic extracts (Rivier et al., 1984).

Relatively few neuropeptides have been found to be stimulatory to TSH release in amphibians. Gonadotropin-releasing hormone (GnRH) stimulated the thyroid axis in axolotl and Rana ridibunda (Jacobs et al., 1988; Jacobs and Kuhn, 1988), and this action of GnRH is direct on the pituitary gland (Denver 1988; Okada et al., 2004). The physiological significance of this finding is currently unknown, since the magnitude of the response is far lower than that achieved with similar doses of CRH (Denver, 1988; Okada et al., 2004). GnRH did not significantly increase TSH release by tadpole pituitary cells in vitro (Okada et al., 2004). Other potential stimulators of TSH by the amphibian pituitary gland include vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP), which have been shown to stimulate TSH secretion by primary pituitary cells from adult bullfrogs (Okada et al., 2007b). The physiological significance of these actions is unknown, as are whether such actions occur in the tadpole.

### 2.09.2.4.4 Negative Feedback Regulation of TSH

As discussed above, the rise in TH production during metamorphosis is driven by pituitary TSH. The synthesis and secretion of TSH are controlled by neurohormones produced by the hypothalamus, and by maturational effects, and negative feedback actions of TH on the hypothalamus and the anterior pituitary gland. The challenge has been to decipher which of these processes account for the sustained rise in TSH (and thus thyroid activity) that occurs during metamorphosis, and the subsequent decline in thyroid activity after metamorphic climax.

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 (Figure 2; Jacobs and Kuhn, 1992; Kaneko et al., 2005). The discovery of a sustained rise in TSH production and thyroid activity during tadpole metamorphosis led William 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 metamorphic tadpole. For example, treatment of premetamorphic tadpoles with thyroid hormone synthesis inhibitors (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 (Goos, 1968; Goos et al., 1968; Dodd and Dodd, 1976; Goos, 1978). Furthermore, goitrogen treatment of premetamorphic tadpoles caused a dramatic elevation in TSHβ mRNA (Buckbinder and 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 and Denver, 2004; Sternberg et al., 2011). Pituitary sensitivity to negative feedback by TH may decline slightly during late premetamorphosis and metamorphic climax, perhaps due to the upregulation of pituitary Dio3 at this time (Manzon and Denver, 2004; Sternberg et al., 2011). Kaneko et al. (2005) found that CRH-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 premorphinomic and early metamorphic 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 et al., 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 and 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 T3 suggests that 5′ deiodinase activity is either present in the pituitary throughout metamorphosis, or the conversion of T4 to T3 is not required for negative feedback. Thyroid hormone receptor β is required for transcriptional repression of the TSHβ and TRH genes in mammals (Flamant and Samarut, 2003; Guissouma et al., 2005). TRβ mRNA increased throughout metamorphosis in the tadpole pituitary (Manzon and Denver, 2004).

Despite the presence of functional negative feedback in the metamorphic 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 metamorphosis 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 (Manzon and Denver, 2004; Kaneko et al., 2005). 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 feed-
back. The relatively lower levels of pituitary Dio2 and TRβ
expression during early prometamorphosis might be permis-
sive for the sustained rise in TSH during prometamorphosis.

2.09.2.4.5 Developmental Expression and Regulation of Adrenocorticotropic Hormone

Expression of proopiomelanocortin (POMC; precursor of
ACTH) mRNA in the anterior pituitary is low in prometamor-
phic tadpoles and increases during prometamorphosis peak-
ning at metamorphic climax (Aida et al., 1999). To my
knowledge there have been no direct measures of adrenocor-
ticotropin hormone (ACTH) during tadpole development.
Functional ACTH receptors are expressed by tadpole
interrenal glands prior to the onset of metamorphosis
(Glennemeier and Denver, 2002a). Premetamorphic tadpoles
are capable of mounting a CORT response in response to a
physical stressor (shaking/confinement stressor)
(Glennemeier and 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 func-
tional maturation of the hypothalamo–hypophyseal–
interrenal axis is reflected in the earlier expression of the
CRH1 receptor (expressed on corticotropes; expression at NF
stage 52) compared with the CRH2 receptor (expressed on
thyrotropes; expression at NF stage 57) (Manzon and Denver,
2004; Okada et al., 2009). The early maturation of the hypo-
thalamo–hypophyseal–interrenal axis provides for environ-
mental stressors to elevate endogenous corticosteroid
production in prometamorphic 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. CRH
and arginine vasopressin (AVP; AVT is the amphibian
hormone) have been shown to stimulate ACTH secretion by
cultured adult frog pituitaries (Tonon et al., 1986).

2.09.2.4.6 Prolactin and Growth Hormone

A central prediction of Etkin’s (1968) model for the endocrine
control of tadpole metamorphosis was that the metamorphic
actions of TH were balanced by the inhibitory actions of the
pituitary hormone prolactin (PRL). Etkin proposed that in
the prometamorphic 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 and Nicoll, 1981), which led
some investigators to suggest that PRL exerted a juvenilizing
action in amphibian larvae similar to juvenile hormone in
insects (Bern et al., 1967; Etkin and Gona, 1967).

The early studies that led to the development of the Etkin’s
model have been extensively reviewed (Dodd and Dodd, 1976;
White and Nicoll, 1983; Kikuyama et al., 1993; Denver, 1996;
Kaltenbach, 1996). 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,
body growth in amphibia as in other vertebrates (Harvey
et al., 1993) has been demonstrated by many studies in which
GH was injected into tadpoles or frogs (White and Nicoll,
1981; Kikuyama et al., 1993; Denver, 1996) and more recently
through transgenic overexpression of GH in X. laevis (Huang
and Brown, 2000a).

By contrast to GH, a role for PRL in regulating tadpole
growth and metamorphosis continues to be controversial
(Huang and 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 metamor-
phosis; the latter action likely through the prevention of TRβ
autoinduction (Tata et al., 1993). Furthermore, 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 or
could be caused by immune reactions to heterologous proteins.
However, it is noteworthy that blockade of endogenous PRL by
passive immunization accelerated metamorphic changes,
which supports a physiological role for the endogenous
hormone (Kikuyama et al., 1993; Denver, 1996). 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 concen-
trations at metamorphic climax. The rise in circulating concen-
trations 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 premeta-
morphosis and also rise, more or less in parallel with TH,
during late prometamorphosis and climax (Clemons
and Nicoll, 1977; Yamamoto and Kikuyama, 1982; Takahashi
et al., 1990; Niinuma et al., 1991b; Buckbinder and Brown,
1993), 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 and Brown,
1993). Similarly, [125I]-PRL binding to kidney membrane frac-
tions was low in premetamorphic bullfrog tadpoles and
increased during metamorphic climax (White and 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 et al.
(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 and Brown, 1993; Huang and Brown, 2000b).
However, Kikuyama et al. (1993) have argued, based on their
experiments with passive immunization with antisera to
bullfrog PRL, that low levels of PRL during the prometamor-
phic/early metamorphic period might be sufficient to
support larval growth and inhibit TH action.

Huang and Brown (2000a,b) 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 et al. (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. Prolactin 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 (White and Nicoll, 1981; Norris and Dent, 1989; Buckbinder and Brown, 1993). The expression of type 2 TRH receptor mRNA in the tadpole pituitary increased through late prometamorphosis and peaked at metamorphic climax (Manzon and Denver, 2004). In mammals PRL secretion is induced by stressors (Cooke et al., 2004; Soares et al., 2006). If a similar regulatory relationship exists in amphibia (e.g., see Lorenz et al., 2009), then it may be that the activation of neuroendocrine stress pathways during metamorphosis functions in the late rise in PRL secretion.

2.09.3 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 timing that it takes to reach the minimum size is determined in part by growth opportunity in the larval habitat (Wilbur and Collins, 1973; Werner, 1986). 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 et al., 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 growth hormones are 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 nucleus 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 (1968) hypothesis.

A striking example of the role of the hypothalamus in controlling metamorphosis, in particular the role of hypothalamic CRH, 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, CRH-like peptides control TSH secretion in tadpoles, acting via the CRH2 receptor. Because the secretion of CRH is activated by stressors, CRH 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) (Denver, 1997a, 1998a; Denver et al., 1998; Boorse and Denver, 2004). The timing of the expression of receptors for neurohormones in the pituitary gland, particularly the CRH2 receptor, could be important in establishing competence of pituitary thyrotropes to respond to stimulation by CRH-like peptides (Manzon and 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 (Glennemeyer and 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–hypophyseal–interrenal axis in mediating the larval developmental response to environmental conditions (Glennemeier and Denver, 2002b). Hayes (1997) also reported an elevation in whole-body CORT content in tadpoles caused by crowding. By contrast, Belden et al. (2007) did not find such a relationship in a mesocosm study (outdoor experimental pools exposed to the natural environment under controlled conditions). 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. An example of indirect regulation of monodeiodinases by environmental factors is by CS, which has been shown to increase 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. Thyroid hormone receptor β is autoinduced in many tissues during metamorphosis, and evidence suggests that this is required to drive metamorphosis (Bagamasbad and Denver, 2011; Laudet, 2011). Hollar et al. (2011) recently showed that TR expression level is negatively correlated with the duration of larval metamorphosis (e.g., cell proliferation vs cell death vs cell differentiation); how the tissues respond asynchronously to the hormone signal to orchestrate appropriate timing of organogenesis and tissue remodeling; and the physiological/developmental mechanisms by which tadpoles respond to environmental variation, among other questions.

As discussed earlier, tadpoles must reach a minimum body size to initiate metamorphosis. Size at transformation varies by several orders of magnitude across amphibian species, but we currently do not know what constrains this size requirement, and how body size is monitored by the animal’s neuroendocrine system. How have the body size (or body condition) thresholds for the initiation of metamorphosis been shaped by natural selection? What are the underlying endocrinological/developmental mechanisms involved? Potential candidate molecules for signaling appropriate energy reserves to initiate metamorphosis are adiposity factors such as leptin (Denver et al., 2011) and insulin, which could function both as developmental and physiological signals to the hypothalamic neurosecretory neurons that control CRH secretion, thereby influencing the timing of metamorphosis.

Other unanswered questions in the field of endocrine control of metamorphosis include:

1. How does the development and activity of endocrine tissues, and hormone action modulate phenotypic plasticity? Current evidence supports that CRH plays a central role in this process and that the CRH₂ receptor mediates the actions of CRH on TSH secretion. However, the role of the CRH₂ receptor in the timing of metamorphosis has not been examined. State-of-the-art gene knockdown and genome editing techniques (e.g., CRISPR/Cas9) can be applied to this question.
2. What are the underlying mechanisms for the developmental ‘decision’ to become reproductively mature or to metamorphose in facultative paedomorphic salamanders? What were the selective pressures that led to the evolution of obligate paedomorphic species, and how has regulation of their HPT axis and/or tissue-specific TH action changed?
3. What evolutionary and developmental mechanisms led to the evolution of direct development in amphibians, and what is the role of TH in these species?
4. What is the role of TH monodeiodination in modulating TH action during metamorphosis? How are the monodeiodinases regulated? How are intracellular hormone metabolite concentrations modulated in the push–pull model of TH metabolism; that is, how does Dio2 and Dio3 cooperate to modulate the intracellular concentration of bioactive hormone? What are the roles of the

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2.09.4 Future Directions

Since Gadernatsch (1912) conducted his experiments that showed that the thyroid gland contained a substance that could accelerate tadpole development, the study of the endocrinological control of amphibian metamorphosis has contributed in diverse ways to basic knowledge of how hormones influence animal development. However, there remain many unanswered questions regarding how hormones modulate developmental processes that occur in different tadpole tissues during metamorphosis (e.g., cell proliferation vs cell death vs cell differentiation); how the tissues respond asynchronously to the hormone signal to orchestrate appropriate timing of organogenesis and tissue remodeling; and the physiological/developmental mechanisms by which tadpoles respond to environmental variation, among other questions.

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monodeiodinases in hormone action, and negative feedback on pituitary TSH secretion? Does the expression and activity of the Dio genes underlie some aspects of asynchronous tissue transformations? What is the role of Dio protein stability and turnover?

5. What are the roles of the plasma TH binding proteins, especially TRα in modulating TH action during metamorphosis?

6. How are plasma membrane TH transporters and intracellular TH binding proteins modulated during metamorphosis and what is their role in timing the metamorphic transition (see Choi et al., 2015a)?

7. Do TRα and TRβ have distinct roles in metamorphosis? Do they regulate different target genes? Recent studies using TALENS to knock out TRα support a role for this receptor subtype in the timing of metamorphosis (Choi et al., 2015b; Wen and Shi, 2015). A deeper analysis of the phenotype displayed by these mutant animals, and generation and analysis of TRβ knockouts may shed light on the developmental functions of these two receptor subtypes.

8. Although there have been several gene expression screens for TH-regulated genes in different tadpole tissues, to my knowledge no studies have looked at the transcriptome during spontaneous metamorphosis in any tissue. Thus, we do not know the extent to which the TH-induced changes reflect normal, developmental patterns of gene expression.

These and other questions may be addressed by scientists fascinated by the dramatic transformation of tadpole to frog.

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