

Hormone regulation and the evolution of frog metamorphic diversity

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7.1 Introduction

A vast body of work exists on the endocrine regulation of frog metamorphosis (Dodd and Dodd 1976, Shi 1999, Denver *et al.* 2002, Buchholz *et al.* 2006, Furlow and Neff 2006, Brown and Cai 2007), and a separate set of literature describes the dramatic larval period diversity found among amphibians (Duellman and Trueb 1994, McDiarmid and Altig 1999). This chapter begins to unite these topics by addressing endocrine and molecular mechanisms underlying life history evolution in larval amphibians. Amphibian metamorphic life history diversity includes species differences in time to, and size at, metamorphosis, evolution of direct developing forms from typical free-living larvae, and neoteny. Ecological aspects of this diversity are not covered, nor is the diversity of reproductive modes. Metamorphic life history parameters are under neuroendocrine control, including both central and peripheral control mechanisms (Fig. 7-1). Despite limited evidence, theory suggests mechanistic changes at the central level of control, whereas evolution of control at the peripheral level has been more adequately demonstrated. We present mechanisms of tissue responsiveness to thyroid hormone from model frog species in order to shed light on potential sites of evolutionary change in endocrine physiology that may underlie life history differences found within amphibians.

The larval period of the amphibian life cycle occurs from the beginning of post-embryogenesis (i.e., completion of organogenesis) through metamorphosis to the production of a juvenile frog. Within tadpoles, life history variation includes larval period duration

(ranging from 8 days in *Scaphiopus couchii* to 2–3 years in *Heleophryne* sp., *Ascaphus*, and some *Rana*) and size at metamorphosis (ranging from <1 cm in many species to adult size in *Pseudis* sp.). Even though virtually every environmental factor affects larval period duration and size at metamorphosis (Wilbur and Collins 1973, Denver 2009), species diversity in larval period and metamorph size is not completely accounted for by phenotypic plasticity, as determined by rearing different species under identical laboratory conditions (Lieps and Travis 1994, Buchholz and Hayes 2002). Direct development and neoteny are dramatic evolutionary departures from the free-living transitory larva. Direct developers lack a free-living larval period and hatch from the egg as a juvenile. Evolutionary loss of larval features in direct developers varies widely across species, from *Eleutherodactylus*, with its vestigial and highly modified larval structures, to other species which hatch from the egg with the appearance of a tadpole but have enough nutrition from yolk to complete metamorphosis (Callery *et al.* 2001, Thibaudeau and Altig 1999). Conversely, neoteny is reproductive maturation in the larval form, found in salamanders but not frogs or caecilians (Dent *et al.* 1968). Depending on the species, neotenic salamanders do or do not undergo metamorphosis in nature, and can or cannot be induced to undergo metamorphosis by hormone injections.

7.2 Ecological context of metamorphic life history evolution

Both time to and size at metamorphosis are important life history traits (Smith 1987). Short larval

period durations are often favored to increase larval survival from aquatic challenges such as predators and pond drying. Larger metamorph sizes are often favored to increase overwinter survival rates and adult reproductive fitness (larger males are often preferred by females and larger females have larger clutch sizes). However, evolutionary changes in larval period may impact metamorph size and vice versa due to an apparent trade-off between time to, and size at, metamorphosis. For example, if pond drying is a relatively large threat then evolution of a short larval period may be accompanied by a decrease in metamorph size because:

1. energy allocation is shifted away from growth to enable more rapid development
2. less time for growth reduces metamorph size (assuming ancestral rates of growth).

The impact of this trade-off could be reduced via evolutionary changes in rates of growth and development (such that size at metamorphosis could be maintained at a shorter larval period). However, the relationship between time to and size at metamorphosis varies widely across amphibians, in that not all rapidly developing species are small, and some slowly developing species are quite small. Thus, knowledge of mechanisms underlying growth and development and the mechanistic relationship between them will help explain the diversity of larval periods and metamorph sizes observed.

7.2.1 Escape from the growth versus development trade-off

Some lineages have radically departed from this paradigm by evolving direct development. Direct developers have “chosen sides” of the trade-off in which relatively small size was an acceptable consequence of eliminating the uncertainties of the aquatic habitat. In contrast to direct development, where development is favored over growth, salamander neoteny is a major life history paradigm, where metamorphic development is abrogated and larval forms become reproductively mature. Neoteny often occurs in cases where the terrestrial habitat is inhospitable or non-existent (as in caves).

7.3 Key concepts in the endocrinology of metamorphosis

7.3.1 Overview of the endocrinology of metamorphosis

Hormones control the morphological and physiological transitions during metamorphosis, including limb elongation, gill and tail resorption, and remodeling of most other organs from the larval to adult versions (Dodd and Dodd 1976). Of predominant importance are thyroid hormones (THs), which induce a gene regulation cascade leading to metamorphic changes (Shi 1999). THs are small molecules derived from two tyrosine residues. The predominant form of TH released into the blood by the thyroid is thyroxine (T4), which has two iodines on each tyrosine moiety, and needs to be altered by the removal of an iodine to become the active form of TH, triiodothyronine (T3). T4 can be considered a hormone precursor compared to T3 because T4 has a 10-fold lower affinity for the TH receptor (TR) compared to T3. Thus, T3 is the form that controls initiation and rate of transformation for each tissue, as well as the overall duration of the larval period. Both T4 and T3 travel in the blood mostly bound to plasma proteins such as transthyretin and albumin. THs enter the cell and bind TR in the nucleus to interact with tissue-specific transcription factors and alter gene transcription associated with metamorphosis. Other hormones, such as corticosterone and prolactin, modulate but cannot replace TH in the control of progress through metamorphosis (Kaltenbach 1996).

All frogs, and indeed all vertebrates, have a peak in TH at some point during their life cycle (White and Nicoll 1981, Buchholz *et al.* 2006). In frogs, this peak in TH occurs at climax of metamorphosis and its regulation is under central control via production of corticotropin releasing factor (CRF), secreted from axon terminals in the median eminence into the pituitary portal circulation and acting on the pituitary (Denver 1996). CRF induces release of two hormones from the pituitary:

- 1) adrenocorticotrophic hormone (ACTH) from corticotropes, which stimulates interrenal glands to produce corticosterone

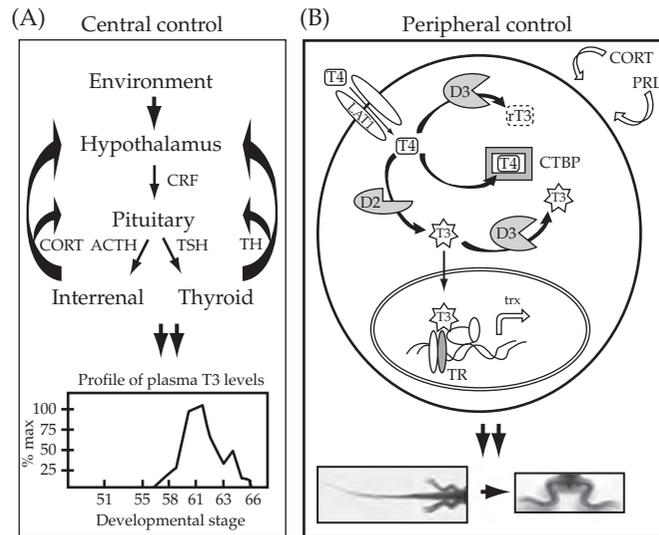


Figure 7-1 Central and peripheral control in thyroid hormone physiology. (A) Brain processing of environmental signals regulates hypothalamic neurosecretion of corticotrophic releasing factor (CRF). CRF enters the pituitary portal vein to induce release of adrenocorticotropic hormone (ACTH) and thyroid-stimulating hormone (TSH). ACTH induces the interrenal glands (homologous to mammalian adrenal glands) to synthesize and release corticosterone (CORT), and TSH acts on the thyroid gland to stimulate release of thyroid hormone (TH). Both CORT and TH exert maturational and feedback effects on the brain, hypothalamus, and pituitary. These regulatory interrelationships constitute central control and give rise to the developmental profile of blood TH titre. The profile for CORT (not shown) resembles that for TH. (B) Circulating TH (mostly in the form of T4) enters the cell via any number of TH transporters (the L-type amino acid transporter LAT1 is shown). Once inside the cell, T4 may be (i) degraded by deiodinase D3 to the inactive TH, reverse T3 (rT3), (ii) sequestered by cytoplasmic thyroid hormone binding proteins (CTBP), or (iii) converted by deiodinase D2 to the active TH, T3. T3 may then enter the nucleus to bind to TR on promoters or enhancers of TH-response genes. T3 binding to TR causes displacement of corepressors for coactivators and subsequent transcriptional activation (trx). CORT and prolactin (PRL) signaling mechanisms (not shown) may affect one or more of the TH transport, metabolism, and transcription processes, thereby modulating gene induction by TH. These mechanisms constitute peripheral control of TH signaling, ultimately resulting in metamorphic changes, exemplified by tail resorption.

2) thyroid-stimulating hormone (TSH) from thyrotropes, which stimulates the thyroid gland to produce TH.

Corticosterone and TH affect this process by maturational actions on the brain and pituitary and by feedback control on the hypothalamus and pituitary. Thus secretion of hormones by the thyroid and interrenal glands is controlled centrally by the hypothalamus and pituitary. Peripheral control refers to cellular and molecular mechanisms regulating the ability of TH/TR complexes to alter gene expression. TRs are ligand-activated transcription factors constitutively bound to enhancers and promoters of TH-response genes. Gene expression and subsequent developmental changes are induced by TH only if free active forms of TH (i.e., T3) can enter the nucleus and bind TR. For cells to respond, the hormone must first enter the cell from the blood via

membrane transporters. Then, deiodinases metabolize THs to active or inactive forms. Cytoplasmic TH-binding proteins can bind T3 in the cytoplasm, perhaps preventing it from entering the nucleus. Peripheral control determines tissue sensitivity and responsivity (see Section 7.3.2) to circulating TH and corticosterone and thereby contributes to the timing of tissue transformation. Because TH physiology regulates developmental timing during metamorphosis, evolutionary changes in central and/or peripheral control may contribute to metamorphic diversity.

7.3.2 Tissue sensitivity and tissue-specific responses to thyroid hormones

Tissue sensitivity refers to whether or not a threshold concentration of TH has been achieved to elicit

a response by the tissue. Tissues differ in their sensitivity to TH at specific developmental stages (e.g., the hind limbs require lower TH levels to initiate transformation compared to the tail). Individual tissues may also vary in sensitivity to TH across development (e.g., at the climax of metamorphosis, the hind limbs are no longer affected by TH). Tissue sensitivity differences may also be found in the same tissue across species. Tissue-specific responses to TH refer to the tissue-specific gene expression and morphological responses to TH, and the rate at which these changes occur. The two concepts of sensitivity and tissue-specific responses are interrelated because some genes that determine TH sensitivity are themselves induced by TH in a tissue-specific manner.

Whether or not a tissue is sensitive to TH resulting in altered gene expression is determined by:

- 1) the amount of TH in the cell nucleus required to achieve a threshold number of hormone-bound TH receptors (TRs)
- 2) the expression TRs, coregulatory factors, other transcription factors, and the activity of other hormonal systems (such as activated corticosteroid receptors).

The threshold effective cellular TH concentration that determines tissue sensitivity involves TH transporters, TH metabolizing enzymes, cytoplasmic TH binding proteins, and TRs and associated transcriptional cofactors (Section 7.5). Above the threshold TH level, higher TH concentrations increase the rate of gene induction and consequent morphological change, up to a maximum level.

7.3.3 Tissue developmental asynchrony

During metamorphosis, each tissue is exposed to the same blood levels of TH yet undergoes remodeling at different times. This tissue asynchrony, where limbs develop first, then intestine remodels, then tail resorbs, is due to tissue-specific initiation and/or rate of transformation in response to TH. Differences in tissue sensitivity and responsiveness among tissues, the gradual increase in TH up to metamorphosis, and the tissue-specific alterations of tissue responses to TH by other hormones such

as corticosterone and prolactin account for tissue asynchrony. Little coordination of timing between tissues occurs during metamorphosis (i.e., tissues do not communicate to coordinate timing). Rather, tissue autonomy in response to TH is the rule. Indeed, organs, including tail, hind limb, and intestine, dissected from the tadpole and cultured *in vitro*, transform in response to TH similarly to their *in situ* counterparts (Derby 1975, Ishizuya-Oka and Shimozawa 1991, Tata *et al.* 1991). In addition, transgenic studies show striking tissue developmental autonomy of tissues, with nerves, muscle, cartilage, and skin of the hind limb transforming independently of one another (Das *et al.* 2002, Marsh-Armstrong *et al.* 2004, Brown *et al.* 2005).

Tissue asynchrony can break down upon treatment with exogenous TH. Lower concentrations of exogenous TH allow for a normal sequence of developmental events, but at an accelerated pace. On the other hand, high levels of exogenous TH lead to initiation of all tissue developmental programs at the same time, even though they would not normally develop synchronously. With high TH, tissue asynchrony is also severely disrupted because some metamorphic events take more time than others. For example, limb elongation, which takes a long time, normally ends before opercular window formation, which occurs quickly. In the presence of high TH, however, opercular window formation is complete before the limbs have completely elongated. The next sections detail endocrine and molecular mechanisms underlying the control of initiation of tissue sensitivity/responsivity associated with derived life histories.

7.4 Endocrine basis of amphibian life history evolution

7.4.1 Larval period duration

The time to metamorphosis (from beginning of feeding to tail resorption) is determined by the time of initiation and rate of metamorphosis. Because TH is necessary and sufficient to initiate tissue remodeling during metamorphosis, much of amphibian metamorphic diversity in larval period duration can be explained by evolutionary changes in central and/or peripheral control

mechanisms of TH physiology. The initiation of metamorphosis depends on developmental maturation of the neuroendocrine system, tissue TR expression, and environmental factors that affect central control (such as pond duration, food availability, and/or predators). Because TR expression occurs very early in the larval period prior to the presence of circulating TH (Baker and Tata 1990), maturation of central control determines the timing of metamorphic initiation. However, the timing of metamorphic initiation is a plastic trait within species, which is subject to brain processing of environmental signals and body condition cues leading to activation of the metamorphic process (Denver 2009). The initiation of metamorphosis varies across species due in part to the rate of embryonic development to achieve maturation of the neuroendocrine system, as well as changes in central control of growth and development rates. Once initiated, the rate of metamorphosis depends both on circulating levels of TH, which are determined by central control, and tissue sensitivity/responsivity to TH, which is determined by peripheral control (see below).

Evolutionarily, it would seem easiest to achieve species differences in metamorphic rate and larval period duration by a single change at the central level (such as a change in CRF production or signaling) to affect the developmental profile of TH and corticosterone. Such a change would make the hormone peaks occur earlier in time and would perhaps achieve higher levels, which would then act globally on all tissues to increase development rate and reduce the larval period. Indeed, hormone levels within tissues have been measured in tail and liver across species in spadefoot toads, showing that species with faster rates of metamorphosis have a higher TH tissue content during metamorphosis (Buchholz and Hayes 2005). However, it is also possible that hormonal differences seen within tissues may not reflect differences in blood concentrations. For example, tissue-specific expression levels of TH-degrading enzymes could result in different amounts of cellular hormone retention despite similar blood TH levels (see below). Thus, the role of central control underlying species differences in larval period duration is not clear. On the other hand, evidence

exists for altered peripheral control as a contributor of species differences in developmental rate. Tail tips of three spadefoot toad species in culture shrank at different rates after exposure to T3, consistent with their differences in larval period (Buchholz and Hayes 2005). Altered larval periods due to changes in peripheral control would seem to require changes in each tissue to avoid disruption of normal tissue developmental asynchrony. Thus, changes in peripheral control are expected to comprise many small evolutionary changes over time.

7.4.2 Size at metamorphosis

Growth hormone (GH) and insulin-like growth factors control size in all vertebrates and transgenic overexpression of GH results in larger tadpoles and adults in the African clawed frog *Xenopus laevis* (Huang and Brown 2000a). Size at metamorphosis may also be controlled indirectly by larval period duration, where environmental signals may alter initiation and/or rate of development, thereby changing the overall time for growth and resulting in different metamorph sizes. Tadpole size and developmental stage are highly correlated, but the extent to which mechanistic interactions between growth and development exist is virtually unknown. However, larval period duration was not affected by transgenic GH overexpression. Thus, any trade-offs that may exist between growth and development are in need of further study.

7.4.3 Direct development

The biphasic amphibian life history of larvae metamorphosing to the juvenile, found in many frog families and plethodontid salamanders, is ancestral to direct developers, which lack free-living feeding larvae. Endocrinological studies on the frog *Eleutherodactylus coqui* have shown:

- similarities in TH titre profile
- CRF release of TSH and ACTH
- lack of TSH release by TRH.

These observations are all consistent with conservation of central control of metamorphosis (Jennings and Hanken 1998, Callery and Elinson

2000, Kulkarni *et al.* 2010). Conserved larval neuroendocrine control mechanisms may suggest that direct development evolved from ancestral indirect development via changes in the peripheral regulation of metamorphosis. Importantly, loss of requirement for feeding in aquatic larval form (except in viviparous caecilians and the viviparous toad *Nimbaphrynoides occidentalis*, where fetal feeding occurs) likely led to the lack of functional larval organs such as larval mouth parts, gills, and intestine. Despite this deletion of a functional larval phase, the ontogeny of direct developers still contains vestigial and non-functional larval organs. Surprisingly, treatment with methimazole, which inhibits TH production, blocked development to the juvenile form of limbs, skin, intestine, muscle, and tail in *E. coqui* (Callery and Elinson 2000). Thus, tissues are still sensitive to TH and depend on TH for development to the adult versions. Therefore, neither central nor peripheral control seems to have changed in a way that would explain the evolution of direct development. Embryonic and tissue-specific development prior to a response to TH seem to be changed, instead of TH physiology itself. Although extensive research in embryonic modifications to deal with a large yolky egg has been done (Elinson 2001), evolutionary changes in the mechanisms of post-embryonic development (i.e., after all major organ systems have been developed), but prior to TH involvement, are unknown.

7.4.4 Neoteny

Reproductive maturation in the larval form is not found in frogs or caecilians but is widespread among salamanders (Duellman and Trueb 1994). Some neotenic salamanders are capable of undergoing metamorphosis in nature under certain environmental conditions. Thus, because peripheral responses to TH leading to metamorphosis are intact, mechanisms of neoteny are to be found in central control. The Mexican axolotl undergoes metamorphosis only when induced by T3 in the laboratory. Surprisingly, the axolotl has a peak in T4 early in life, when toes differentiate, but metamorphosis fails to occur (Rosenkilde *et al.* 1982). Lack of a peripheral deiodinase conversion of T4 to T3, in combination with

low TR levels, may explain neoteny in axolotls (Galton 1992, Rosenkilde and Ussing 1996).

The obligate neotenic salamander *Necturus maculosus* cannot undergo metamorphosis even when treated with TH although TRs are present, functional *in vitro*, and expressed *in vivo* (Vlaeminck-Guillem *et al.* 2006). In *Necturus*, loss of TH-induction of key genes involved in metamorphosis may explain its neoteny. Thus, the molecular mechanisms underlying neoteny vary with the type of neoteny and may be found at central and/or peripheral levels.

7.5 Molecular mechanisms of peripheral control: Potential evolutionary targets underlying diversity in larval period diversity

Even though changes in central control may have contributed to amphibian metamorphic diversity, we focus here on peripheral control (Fig. 7-1B), where potential evolutionary changes are better known. The timing of transformation for each tissue is determined by the combinatorial effect of TH signaling proteins expressed at their tissue-specific level. Evolutionary changes in the expression and activity levels of these proteins can change tissue sensitivity/responsivity to TH and can thereby alter the timing of initiation and/or rate of tissue transformation, leading to species differences in larval period duration. Below, proteins involved in TH transport, metabolism, and gene regulation in frogs are described, including their potential role underlying amphibian life history diversity.

7.5.1 Thyroid hormone transporters

Many transport proteins that allow TH entrance into the cells have been identified. These have varying efficiency and specificity for TH and have been sorted into different families (Heuer and Visser 2009). These proteins transport THs as well as other small molecules, often amino acids. MCT8 and OATP1c1 (monocarboxylate anion transporter 8 and organic anion transport protein 1c1) described in mammals have the highest specificity for TH among all TH transporters and preferentially transport THs across cell membranes. Importantly, TH transport has the potential to

regulate TH bioavailability in the nucleus because TH transport is specific, subject to regulation, and rate-limiting for intracellular TH metabolism (Hennemann *et al.* 2001). The strongest evidence for an *in vivo* role of TH transport comes from the association of mutations in MCT8 with severe X-linked mental retardation and elevated circulating T3 levels (Jansen *et al.* 2008). Most TH transporters are expressed in varying patterns in different types of tissues (Heuer and Visser 2009). *In-vitro* studies have shown that the activity of these transporters affects tissue sensitivity to TH and how the cell responds to competing substrates like amino acids (Shi *et al.* 2002).

The only TH transporter studied in frogs is the L-type amino acid transporter 1 (LAT1). Expression of LAT1 varies across tissues and correlates with tissue transformation during metamorphosis (Shi *et al.* 2002). An *in-vivo* role for LAT1 is suggested by its strong up-regulation during TH-dependent development (Liang *et al.* 1997). In addition, LAT1 overexpression in *Xenopus laevis* oocytes affected TH-response gene transcription (Ritchie *et al.* 2003). Because the free serum TH concentration is 1000 times lower than the receptor binding constant (K_m) for LAT1, TH bioavailability via LAT1 is not regulated by substrate saturation, but rather by the number of transporters in the cell membrane. Thus, changes in LAT1 expression across species may affect tissue sensitivity, thereby potentially altering metamorphic initiation. However, at least two features of LAT1 function may limit the potential role of LAT1 in metamorphic diversity. First, LAT1 cannot function without its heterodimerization partner, 4F2 (Friesema *et al.* 2001), and thus, simultaneous and/or sequential evolutionary changes in both LAT1 and 4F2 may be required for this TH transporter to affect TH bioavailability across species. Second, ligands for LAT1 include not only TH, but also leucine and large aromatic amino acids (tryptophan, phenylalanine, tyrosine), such that changes in the flux of these amino acids could occur and may disrupt normal cellular function.

7.5.2 Thyroid hormone metabolizing enzymes

Like mammals, three deiodinases, D1–3, have been identified in amphibians (St Germain *et al.* 1994, Davey *et al.* 1995, Kuiper *et al.* 2006). Developmental

studies in amphibians focused on D2 and D3 because frogs were not known to have D1 until recently (Kuiper *et al.* 2006). D1 activity in frogs had not been detected biochemically because, unlike the mammalian version, it is not inhibited by propylthiouracil. D1 converts T4 to T3, T4 to reverse T3 (rT3, an inactive form), and T3 and rT3 to T2 (another inactive form). D2 activates T4 to make T3, and D3 inactivates T4 and T3 to rT3 and T2, respectively. These enzymatic activities are significant because only T3 has biologically significant affinity to TR.

D2 and D3 act to regulate tissue developmental asynchrony during metamorphosis (Brown 2005). Studies measuring deiodinase activity in *Rana catesbeiana* showed that high rates of T4 to T3 conversion correlated with the timing of transformation of limbs, intestine, skin, and eye (Becker *et al.* 1997). *In-situ* hybridization in *X. laevis* confirmed the presence of D2 in transforming tissues (Cai and Brown 2004). Experimental evidence for the importance of D2 in regulating the timing of differentiation was shown by use of the TH synthesis blocker, methimazole, and the pan-deiodinase inhibitor, iopanoic acid (Becker *et al.* 1997). When tadpoles of *R. catesbeiana* were treated with methimazole and iopanoic acid, hind-limb growth and change in body morphology were inhibited. Hind-limb growth was restored when tadpoles were treated with T3 but not with T4. This confirms that the D2 activity to convert T4 to T3 can control the timing of tissue transformation. In addition, overexpression of D3 using transgenesis has confirmed the ability of D3 to inhibit metamorphic changes in *X. laevis* (Huang *et al.* 1999, Marsh-Armstrong *et al.* 1999).

Species differences in expression and activity of deiodinases could account for differences in larval period duration. For example, tails might shrink faster by expressing more D2 and less D3 in one species compared to another. Also, unlike several other factors that affect TH signaling, deiodinases are specific to TH physiology and would not be burdened by pleiotropic effects on other physiological systems.

7.5.3 Cytosolic thyroid hormone binding proteins

After first being distinguished from serum TH binding proteins (Tata 1958), the existence of cytosolic

thyroid-hormone binding proteins (CTBPs) is now well established and many have been reported in mammals and amphibians (Kato *et al.* 1989, Ishigaki *et al.* 1989, Ashizawa *et al.* 1991, Shi *et al.* 1994, Yamauchi and Tata 1994). Multiple types of CTBPs exist in a given species and are expressed in a variety of tissues. Surprisingly, CTBPs do not represent a specific protein family; they are enzymes of diverse function that seem to have acquired TH binding capacity independently. In frogs, three CTBPs have been identified: aldehyde dehydrogenase 1, pyruvate kinase subtype M2, and protein disulfide isomerase (Shi *et al.* 1994, Yamauchi and Tata 1994, 1997). Another CTBP in frogs was detected by photoaffinity labeling and SDS-PAGE, but was not identified (Yamauchi and Tata 1997). Yet another CTBP has been reported in *Rana catesbiana* and was characterized as metal-ion-dependent but its identity was not determined (Yoshizato *et al.* 1975).

CTBPs have been shown to affect TH-regulated gene transcription, most likely by modulating intracellular bioavailability of TH (Ashizawa and Cheng 1992, Mori *et al.* 2002) and/or transporting TH to the nucleus (Ishigaki *et al.* 1989). In turn, CTBPs may contribute to developmental tissue asynchrony via decreased tissue-specific expression levels of CTBPs in tissues actively undergoing TH-dependent remodeling (Shi *et al.* 1994). Also, high ALDH1 mRNA expression was observed in the liver and smaller amounts were detected in head, intestine, and tail during metamorphosis (Yamauchi and Tata 1997).

Despite these potential roles in TH physiology, CTBPs are multifunctional proteins, and the primary role of TH binding may be to modulate the activity of the enzyme rather than modulate TH bioavailability. Importantly, the enzymatic function and TH binding of CTBPs are mutually exclusive, i.e., when TH is bound the enzyme is inactive. Furthermore, the equilibrium dissociation constants (K_d) among known CTBPs for TH is often above the concentration of TH found in the blood, indicating that their identification as CTBPs may be of only pharmacological importance and not relevant for physiological TH levels. Thus, the evolution of CTBP expression and function may have been constrained by their enzymatic functions, suggesting they may not be likely candidates for the evolution of differential tissue sensitivity to TH. While *in-vitro*

studies have confirmed TH binding in cytosolic extracts of various tissues (Yamauchi and Tata 1997), and correlations exist between TH binding activity and expression levels of TH response genes (i.e., TR β) (Ashizawa and Cheng 1992, Mori *et al.* 2002), future studies are required to clarify the role of CTBPs in TH-dependent development.

7.5.4 Thyroid hormone receptors

Frogs, like all vertebrates, have two types of TR, TR α and TR β , coded for by two separate genes (Yaoita *et al.* 1990). TRs belong to the nuclear receptor superfamily of transcription factors and influence transcription by binding to TH-response elements (TREs) in DNA. TRs can bind to TREs weakly as monomers or homodimers, but with a much higher affinity as a heterodimer with retinoic X receptors (RXRs) (Wong and Shi 1995). Gene regulation by TRs is hormone dependent, such that in the absence of TH, TRs function to repress expression of TH-response genes, while in the presence of TH they activate those same genes (Buchholz *et al.* 2006). This dual function is achieved through the interaction of TRs with cofactors. Unliganded TRs are bound to corepressors, such as N-CoR (nuclear receptor corepressors) and SMRT (silencing mediator for retinoid and thyroid hormone receptors). These corepressors form complexes with other proteins that function to prevent transcription at promoters of TH-response genes via histone deacetylation. The presence of TH causes a conformational change in TR favoring binding of coactivators, such as steroid receptor coactivator (SRC) 1–3 and p300, leading to the recruitment of additional cofactors, causing changes in histone acetylation and gene induction.

The importance of TRs and co-factors in tadpole metamorphosis is supported by studies using transgenic overexpression of mutant receptors and co-factors (Shi 2009). Metamorphic transformation can be initiated in the absence of TH in tadpoles that overexpress a constitutively active TR (Buchholz *et al.* 2004), and TH-induced morphological changes are accelerated by overexpression of wild-type co-activator protein arginine methyltransferase 1 (PRMT1), which methylates histones and other nuclear proteins (Matsuda *et al.*

2009). On the other hand, none of these developmental events occur in tadpoles overexpressing dominant negative TRs or cofactors, that is, mutant versions that block function of endogenous TRs (Buchholz *et al.* 2003, Paul *et al.* 2005a, 2007). Transgenic tadpoles that overexpressed the dominant negative corepressor NCoR experienced derepression of TH-response genes as well as accelerated premetamorphic development (Sato *et al.* 2007). These studies provide *in-vivo* evidence for the profound role that TRs and co-factors play in gene regulation *in vivo* and in regulating timing of tissue transformation.

The dual molecular function of TRs coupled with their peak in expression during metamorphosis has the potential to impact developmental timing in frogs. TR α , with significant premetamorphic expression levels, is important for repressing TH-response genes to ensure the tadpole does not develop precociously (Sato *et al.* 2007). At the same time, TR α expression levels allow tissues to establish tissue competence to respond to the hormone signal. Because TR β is a direct TH-response gene, it requires TH for significant expression and consequently may be less important than TR α for repression during premetamorphosis and controlling tissue sensitivity. However, premetamorphic tissues contain limiting amounts of TR, and thus autoregulation of TR β expression is necessary for tissues to respond fully to circulating TH (Buchholz *et al.* 2005). Therefore, both TRs participate in the activation of TH-response genes and thus responsiveness to TH. Consequently, evolutionary changes in TR α and TR β expression levels across development and tissues are expected to underlie species differences in metamorphic timing.

7.5.5 Modulation of thyroid hormone responsiveness by corticosterone and prolactin

While TH may be necessary and sufficient to initiate tissue transformation, other hormones interact with TH to affect the timing of developmental events. The blood titre of corticosterone (CORT) and prolactin (PRL) peak at metamorphic climax, like TH (Clemons and Nicoll 1977, Krug *et al.* 1983, Jolivet-Jaudet and Leloup-Hatey 1984), and these hormones can influence the initiation and rate of tissue

transformation (Kaltenbach 1996). However, neither hormone by itself can initiate metamorphic progress. Rather, CORT and PRL have a complex and tissue-specific role, interacting with TH, and underlying tissue developmental asynchrony.

7.5.5.1 Corticosterone

Although TH is sufficient to initiate metamorphosis, TH alone cannot complete metamorphosis in the absence of CORT. Because CORT does not have metamorphic actions independent of TH, the role of CORT is likely to permit a maximal level of TH signaling required to complete metamorphosis. Hypophysectomized tadpoles treated with T4 do not completely resorb the tail nor leave the water, whereas injection of ACTH to induce CORT production allows complete metamorphosis of T4-treated hypophysectomized tadpoles (Remy and Bounhiol 1971). Similarly, bullfrog tadpoles treated with amphenone B, a corticoid synthesis blocker, show inhibited metamorphosis (Kikuyama *et al.* 1982), but a 50% reduction of CORT whole-body levels by the 11 β -hydroxylase inhibitor, metyrapone, does not affect developmental timing (Glennemeier and Denver 2002).

On the other hand, CORT will either inhibit metamorphosis (if present in early stages) or accelerate metamorphosis (if present during later stages). CORT levels increase naturally in the presence of stress and studies have examined the affect of exogenous CORT on developmental timing of amphibian metamorphosis (Leloup-Hatey *et al.* 1990). However, this acceleration by CORT occurs in some tissues, such as tail, but not others, such as hind limbs (Hayes 1995). Generally, CORT has inhibitory actions on growth in all vertebrates, even in prometamorphic tadpoles. CORT's early inhibition of development is unlikely to be via negative feedback on CRH because the receptors for CRF on thyrotropes are not expressed until prometamorphosis (Manzon and Denver 2004). However, by prometamorphosis CORT's role as a facilitator of TH action becomes significant because TH levels are rising. In particular, CORT increases sensitivity and responsiveness to TH in tail tips (Kikuyama *et al.* 1993). CORT affects TH physiology by at least two mechanisms: the regulation of deiodinases and the presence of a glucocorticoid response element (GRE) in the promoter of TR β and perhaps

other TH response genes. Because nearly all cell types express the CORT receptor (GR), the tissue-specific actions of CORT are likely explained by tissue-specific factors affecting gene regulation by GR. CORT enhances T4-to-T3 conversion by increasing D2 activity and decreases the degradation of T3 by reducing D3 activity (Galton 1990), thereby increasing the intracellular concentration of T3. The mechanisms by which CORT affects D2 and D3 are not known, but could be direct via gene regulation or indirect via regulation of genes that affect deiodinase enzyme stability or activity. A GRE may be present in the TR β promoter because CORT upregulates TR β in the intestine (Krain and Denver 2004), so TR β upregulation in the presence of TH and CORT could be due to a combination of CORT's action on TH signaling via D2 and D3 and direct transcriptional regulation at the TR β promoter.

7.5.5.2 Prolactin

At one time PRL was believed to be a juvenile hormone analogous to juvenile hormone in insects because of the anti-metamorphic effects of PRL. However, the developmental expression profile of PRL mRNA in pituitary (Takahashi *et al.* 1990, Buckbinder and Brown 1993), and blood levels of PRL that peak at climax, like TH, in *Bufo japonica* (Niinuma *et al.* 1991) and bullfrog (Yamamoto and Kikuyama 1982) put this interpretation of the role of PRL into question. Functional analysis of PRL via overexpression in transgenic animals shows that PRL indeed antagonizes TH action in transgenic animals, resulting in a tailed juvenile frog, where muscle still resorbs but notochord, fibroblasts, and epithelium remain (Huang and Brown 2000b). However, the transgenic animals otherwise metamorphose at the same time, with no known delays in other organs. PRL receptors are detected in most tadpole tissues tested, with high expression in tail and low expression in liver and intestine (Hasunuma *et al.* 2004). As for CORT, these data argue for tissue-specific action of PRL because, even though PRL receptors are present in many tissues, delays in tissue transformation in PRL overexpressing transgenic tadpoles are observed only in the tail. One mechanism by which PRL antagonizes TH action is upregulation of D3 mRNA in the tail (Shintani *et al.* 2002). The effect of PRL on T3-induced tail shrink-

age is reversed by iopanoic acid, which blocks deiodinase activity. PRL blocks TR β upregulation by T3 (Baker and Tata 1992), where TR β is a direct response gene in no need of protein synthesis, which suggests that PRL signaling can act upstream of TR-mediated gene regulation, in addition to D3 gene regulation.

7.6 Conclusions

Amphibian larval period diversity includes time to and size at metamorphosis, direct development, and neoteny. TR and associated cofactors function as a metamorphic switch and depend upon upstream TH transport and metabolism to regulate the timing and rate of transformation in a tissue-specific manner, contributing to the total larval period duration. Few studies have attempted to identify evolutionary changes in TH signaling that may serve as a mechanistic basis for life history differences among species. Research to understand the control of metamorphosis in model species reveals a large complexity of potential mechanisms from which evolution could "choose" to generate species life history differences. Changes at the central and/or peripheral levels could underlie life history differences, but the available evidence so far has provided more support for peripheral mechanisms. In theory, a change in a single protein that influences TH bio-availability could underlie differences between species, but species differences are more likely controlled by a number of different mechanisms, such as expression and activity changes in TH transporters, CTBPs, TRs, and/or deiodinases. For instance, higher TH sensitivity could be due to increased LAT1, TR, and/or D2 expression or decreased D3 and/or CTBP levels. The dual functionality of CTBPs and multiple substrates for LAT1 may limit evolutionary flexibility in their expression levels and thus may limit their role in contributing to metamorphic diversity. On the other hand, TRs and deiodinases are specific to TH physiology, such that changes in their expression levels might not affect other aspects of development. Future comparative studies to identify specific differences in these proteins between species will require analysis of protein activities and/or cloning relevant genes from each of the derived species for analysis of gene expression pat-

terns. However, such comparative analysis will at best provide correlations between life history traits and expression-level differences of TH signaling genes. Strong inferences about the mechanistic basis of life history divergence will come from experimental tests of these correlations by studying the effects of altered gene expression levels on metamorphosis in model species.

7.7 Summary

1. Amphibian larval period diversity includes time to and size at metamorphosis, direct development, and neoteny.
2. Thyroid hormone physiology regulates the timing and rate of metamorphic transformation on a tissue-by-tissue basis via thyroid hormone receptors and associated cofactors, which together function as a developmental switch dependent upon bioavailability of thyroid hormone in the nucleus.
3. Thyroid hormone bioavailability in the cell nucleus is controlled by cellular mechanisms of thyroid hormone transport and metabolism involving thyroid hormone transporters, cytosolic

thyroid hormone binding proteins, thyroid hormone receptors, and iodothyronine deiodinases.

4. Larval period evolution likely involved changes in expression level or activity of proteins that control thyroid hormone bioavailability.

5. Thyroid hormone receptors and deiodinases are specific to thyroid physiology, such that changes in their expression or activity levels might not affect other aspects of development.

6. On the other hand, the dual functionality of cytosolic thyroid hormone binding proteins and multiple substrates for thyroid hormone transporters may limit evolutionary flexibility in their expression levels and thus may limit their role in contributing to metamorphic diversity.

7. Comparative studies to identify specific differences in proteins controlling thyroid hormone bioavailability between species will require analysis of protein activities and/or cloning relevant genes from each of the derived species for analysis of gene expression patterns.

8. Comparative analyses should be complemented with functional studies on the role of these genes in development using model species.