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Endocrinology of Complex Life Cycles: Amphibians

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I. INTRODUCTION TO COMPLEX LIFE CYCLES

Amphibians exhibit considerable diversity in behavioral, physiological, and life history strategies. They are geographically widespread, occupying a diverse range of habitats. Amphibians that undergo metamorphosis have two very different life stages that are affected differently by environmental factors. Most anuran (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).

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, and photoperiod) as well as biotic factors (e.g., intra- and interspecific competition, and predation) can interact in complex ways to influence larval growth and development (Semlitsch, 1987a; Sredl and Collins, 1992; Rowe and Dunson, 1995; Taylor and Scott, 1997). The timing of metamorphosis is a central amphibian life history trait that probably 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 (see Fig. 1). **Fig. 1**

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; see Newman, 1992). Such plasticity may permit amphibian larvae to match their phenotype (morphology, physiology, and metamorphic timing) to prevailing environmental conditions. Animals capable of phenotypic plasticity may have a higher probability of surviving in unpredictable habitats than those with a genetically fixed phenotype (Stearns, 1989; Newman, 1992).

Among the most extreme evolutionary modifications of the ancestral complex life history is paedomorphosis.

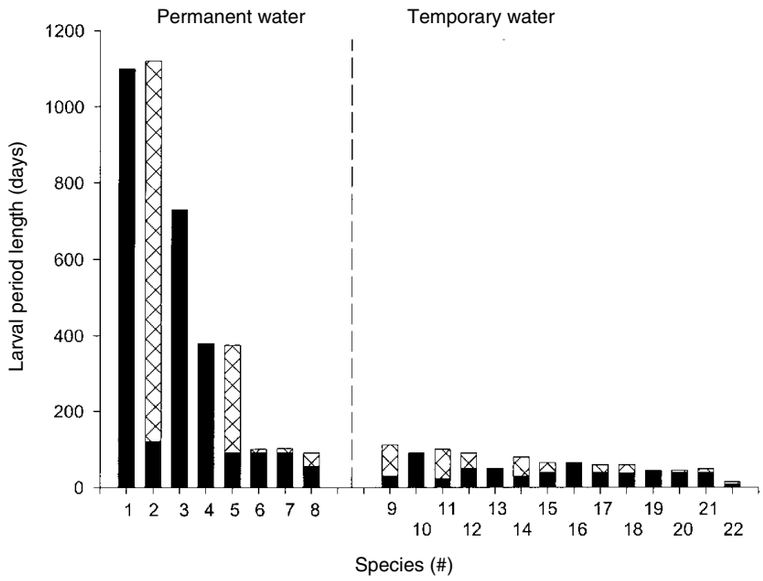

FIGURE 1 The duration of larval periods of selected amphibian species as a function of habitat permanence. Filled bars indicate the minimum duration and hatched bars the maximum duration of the larval period for those species for which information is available. This figure is based partly on information compiled by Low (1976) and Duellman and Trueb (1994) and Denver (1997b). (1) *Rana fuscigula* (Wager, 1965), (2) *R. catesbeiana* (Stebbins, 1951; Bruneau and Magnin, 1980), (3) *R. fasciata fuellborni* (Stewart, 1967), (4) *Ascaphus truei* (Noble and Putnam, 1931; Stebbins, 1951), (5) *R. clamitans* (Stebbins, 1951), (6) *R. grayi* (Wager, 1965), (7) *R. boylei* (Stebbins, 1951), (8) *R. pipiens* (Stebbins, 1951), (9) *R. temporaria* (Miaud *et al.,* 1999; Brady and Griffiths, 2000), (10) *R. sylvatica* (Stebbins, 1951), (11) *Spea (Scaphiopus) hammondii* (Morey and Janes, 1994; R. J. Denver, unpublished data), (12) *Bufo americanus* (Wilbur, 1987), (13) *Pixicephalus adspersus* (Stewart, 1967), (14) *B. boreus* (Hayes *et al.,* 1993), (15) *Hyla arenicolor* (Stebbins, 1951), (16) *H. pseudopuma* (Crump, 1989a), (17) *B. punctatus* (Stebbins, 1951), (18) *B. woodhousei* (Mayhew, 1968; Blair, 1972), (19) *S. bombifrons* (Stebbins, 1951), (20) *B. rangeri* (Stewart, 1967), (21) *B. cognatus* (Stebbins, 1951), and (22) *S. couchi* (Newman, 1988).

Most amphibian larvae undergo a metamorphosis to an adult form before becoming sexually mature. Some species of urodele amphibians (e.g., salamanders and newts) exhibit paedomorphosis, in which 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). Many terms have been used describe sexual reproduction while retaining larval characteristics. We choose to use the term "paedomorphosis" for our discussion because it describes retention of larval traits in a sexually mature form, but does not describe the process by which this state is achieved. Other terms such as "neoteny" (deceleration of somatic development) and "progenesis" (acceleration of sexual maturation) describe processes by which paedomorphic development occurs (for more on terminology, see Gould, 1977; McKinney and MacNamara, 1991; Reilly *et al.,* 1997).

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; Amphiuma;* and *Ambystoma mexicanum,* axolotl). The primary focus

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of our discussion is on facultative paedomorphs, with limited treatment of obligate paedomorphs. 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, A. talpoideum, A. gracile,* and *Notophthalmus viridescens;* Duellman and Trueb, 1994). The developmental decision to become paedomorphic or to metamorphose may depend on the prevailing environmental conditions rather than the animal's genotype (Harris, 1987; Semlitsch, 1987a; Licht, 1992; Jackson and Semlitsch, 1993) and may be controlled by the interplay of antagonistic hormonal pathways (see Sections IV and V).

II. EVOLUTIONARY ECOLOGY OF AMPHIBIANS

A. Metamorphosis

Amphibians exhibit considerable inter- and intraspecific variation in the duration of the larval period. The rate of development generally is inversely related to larval growth rate and therefore to size at metamorphosis, which can have profound effects on individual fitness. Both a longer larval period and a smaller size at metamorphosis can delay adult reproductive maturity, decrease size at first reproduction, and in some cases decrease adult survival to first reproduction (Berven and Gill, 1983; Smith, 1987; Semlitsch *et al.,* 1988). All of these factors decrease the chance of contributing offspring to the next generation. A longer time to metamorphosis may also increase larval exposure time to aquatic predators (Wilbur, 1980; Werner, 1986) or decrease the chance of metamorphosing before a quickly drying pond disappears (Newman, 1992).

1. Environmental Factors That Influence the Duration of the Larval Period

The upper and lower limits of 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 the proximate and ultimate levels by the environment. Although 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 later).

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 habitats to the list of factors that ultimately influence metamorphosis. Environmental factors that influence growth rate or mortality risk therefore should alter the timing of metamorphosis. The effects of specific environmental factors may differ depending on the animal's stage of growth or development. 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.

The predictability of rainfall (and, thus, pond duration) profoundly influences amphibian life history strategies. Species that breed in habitats that are permanent and predictable (i.e., lakes, streams, and permanent ponds) generally have longer larval periods, whereas those that breed in habitats that are unpredictable and ephemeral (i.e., temporary ponds) exhibit rapid development (Fig. 1). A short development time is of particular importance in adaptation to a desert environment in which rainfall is unpredictable and ponds are of short duration (Low, 1976; Newman, 1992).

2. Evolution of the Timing of Metamorphosis

The larval stage often is more vulnerable than the adult stage and may be characterized by a higher degree of uncertainty with regard to individual mortality (Duellman and Trueb, 1994). Tadpoles (and eggs) are more vulnerable than adults to predation, due to their small size and relative lack of mobility (Duellman and Trueb, 1994). Competition for resources may also be especially high among larvae, due to rapid growth rates (and, thus, high energy demands) and high densities of conspecifics. Such competition may increase larval mortality rates (Wilbur and Collins, 1973; Smith, 1983). Because of their aquatic habitat, larvae are also

especially vulnerable to changes in rainfall or humidity levels and the duration of ponds.

When survivorship in one life-cycle stage is much less certain than in another, we expect to see, among other responses, the minimization of the time spent in the more vulnerable stage (Low, 1976). This expectation is confirmed in habitats with unpredictable rainfall and standing water levels, such as those found in arid regions. Desert amphibians generally exhibit rapid rates of development, with some larvae entering metamorphosis in as little as 8 days after hatching (*Scaphiopus couchii;* see Newman, 1992). Spadefoot toads (genus *Scaphiopus*) typically inhabit arid regions and breed in temporary ponds of unpredictable duration. The length of the larval stage in Couch's spadefoot toads typically ranges from 8 to 15 days, compared to a range of 30 to >1000 days for species found in more permanent aquatic habitats (Fig. 1).

The larval stage is typically more vulnerable and uncertain than the adult stage—why haven't all anurans evolved to minimize the time spent in the larval stage? The answer probably involves the trade-off between development rate and size at metamorphosis. Tadpoles that develop rapidly are typically smaller than those that develop more slowly, with a longer period for growth. Small size at transformation may reduce reproductive potential (Berven and Gill, 1983; Smith, 1987; Semlitsch *et al.,* 1988), a cost that limits the benefit of rapid development in an unpredictable larval habitat. In addition to the costs of rapid metamorphosis, physical constraints may also limit growth and development. For example, Wilbur and Collins (1973) proposed that larvae must reach a minimum body size in order to metamorphose.

a) Phenotypic Plasticity Phenotypic plasticity in development time allows larvae to develop either slowly or rapidly, depending on environmental conditions. Phenotypic plasticity refers generally to phenotypic variation induced by environmental change, and a plastic reaction norm, as described by Stearns (1989), refers to the relationship between phenotypic variation and the environment when the phenotype varies as a continuous function of the environmental signal.

Phenotypic plasticity is especially pronounced in desert-dwelling species (and those of other ephemeral, unpredictable habitats). By artificially altering pond duration, Newman (1988) found that *S. couchii* larvae developed faster (and metamorphosed at a smaller size) in short-duration ponds than in long ones. Newman (1988, 1992) concluded that phenotypic plasticity may have developed in these desert anurans as a result of the fitness trade-off between rapid and slow development under various environmental circumstances.

Other species, both desert and nondesert, that breed in unpredictable habitats show developmental plasticity in response to pond drying (see Table 1). Not all species that have been examined respond to pond drying, which may reflect the relative permanence of the ancestral habitat of the species under study. For example, *R. utricularia,* which breeds in more permanent water, did not show accelerated development rate in response to pond drying (Wilbur, 1987; note that *B. americanus* showed a pond-drying response in the same study). Tadpoles of other ranid species showed developmental responses to pond desiccation (see Table 1). Of those that have been studied, more species than not

Table 1

TABLE 1
Amphibian Species That Accelerate Metamorphosis in Response to Pond Desiccation[a]

Species	*Source*
Ambystoma spp.	
A. talpoideum	Semlitsch and Gibbons (1985); Semlitsch (1987a); Semlitsch and Wilbur (1988)
Bufo spp.	
B. americanus	Wilbur (1987)
B. maculatus	Spieler (2000)
Hyla spp.	
H. pseudopuma	Crump (1989a)
Rana spp.	
R. blairi	Parris (2000)
R. sphenocephala	Parris (2000)
R. temporaria	Loman (1999); Laurila and Kujasalo (1999); Merila *et al.* (2000)
Scaphiopus spp.	
S. couchii	Newman (1989); Morey and Janes (1994)
S. hammondii	Denver *et al.* (1998)

[a] Pond desiccation refers to experimental paradigms that include outdoor experiments with cattle tanks and artificial ponds and observations in natural ponds and aquaria maintained in laboratories in which the water level was manipulated. See Brady and Griffiths (2000) for conflicting results.

appear capable of responding to habitat permanence by altering their rates of development (but see Brady and Griffiths, 2000; Spieler, 2000).

To identify the proximate environmental cue(s) that tadpoles of the western spadefoot toad *(S. hammondii)* use to accelerate development in response to pond drying, we manipulated water levels in aquaria in which tadpoles were reared (Denver *et al.,* 1998). Under these laboratory conditions, tadpoles accelerate metamorphosis as the water volume is reduced. Tadpoles can grade their developmental response with respect to the rate of water volume reduction. Furthermore, tadpoles responded to the release from ecological stress (the refilling of the aquarium) by capitalizing on the improved growth conditions. The physiological response to experimental water-volume reduction results in the activation of the endocrine axes that drive metamorphosis (Denver, 1998).

Intraspecific competition may also affect larval development rates. Several studies have shown that resource limitation influences amphibian development, but that the direction of this influence depends on the developmental stage at which the limitation is initiated. For example, increased competition for resources, present early in the larval period, has been shown to decrease larval growth rate, survivorship, and size at metamorphosis and to increase the length of the larval period (Brockelman, 1969; Wilbur and Collins, 1973; Wilbur, 1976, 1977; Smith, 1987; Berven and Chadra, 1988; Scott, 1990). Similarly, D'Angelo and colleagues (1941) showed that starvation before the early limb development stage retarded metamorphosis in both *R. sylvatica* and *R. pipiens;* however, starvation after this stage accelerated metamorphosis. We observed a similar, developmental-stage-dependent phenomenon in *S. hammondii* tadpoles (Denver *et al.,* 1998), and Morey and Reznick (2000) reported nearly identical results in three species of spadefoot toads, *S. couchii, S. hammondii, and S. intermontanus*. These studies suggest that there is a critical period of development for responding positively to limited resources that may reflect a minimum required size or developmental stage for metamorphosis (see also Crump, 1989b; Newman, 1994).

Abiotic factors such as temperature, photoperiod, dissolved oxygen content (DOC), and pH also influence the length of the larval period in amphibians (Wassersug and Seibert, 1975; Gutierrez *et al.,* 1984; Feder and Moran, 1985; Wright *et al.,* 1986; Burns *et al.,* 1987; Edwards and Pivorun, 1991). Increased temperature is well known as accelerating larval growth and development (Hayes *et al.,* 1993). However, temperature can interact in complex ways with other factors such as resource level and density to affect time to and size at metamorphosis (Marian and Pandian, 1985; Beachy, 1995; Newman, 1998).

As mentioned, the environment has a strong influence on the timing of metamorphosis. The physiological bases for plasticity in the timing of metamorphosis are discussed in Section III and placed into an ecological context in Section V.

B. Facultative Paedomorphosis

In facultatively paedomorphic species, both paedomorphic and metamorphic individuals often coexist in the same population in nature and each morph is probably associated with discrete fitness-related consequences. Several hypotheses have been proposed for the maintenance of these alternate morphologies (see Whiteman, 1994, for review), but evidence supports the paedomorphic advantage hypothesis, which predicts that paedomorphosis evolves in relatively permanent, aquatic habitats. In such conditions, paedomorphs experience an advantage in one or more fitness components that lead to increased lifetime reproductive success. In support of this hypothesis, paedomorphs are more prevalent in stable aquatic conditions, such as permanent water (Semlitsch, 1987a) and low larval population density (Harris, 1987; Semlitsch, 1987a); paedomorphs may also be favored in habitats where predation risk is low (Jackson and Semlitsch, 1993) (Fig. 2). **Fig. 2**

Age at maturation is a central life history trait. Paedomorphs undergo sexual maturation earlier than metamorphs (Ryan and Semlitsch, 1998). For example, in Alpine newts *(Triturus alpestris),* metamorphs typically require several years to mature (Miaud *et al.,* 2000), whereas paedomorphs can mature at 1 year of age (Denoel and Joly, 2000). The primary advantages of earlier maturation are greater probability of survival to first reproduction, shortened generation time, and potential increases in lifetime reproductive success (Stearns, 1991; Roff, 1992).

In addition, paedomorphs are present at the breeding site as soon as they mature, allowing them to reproduce

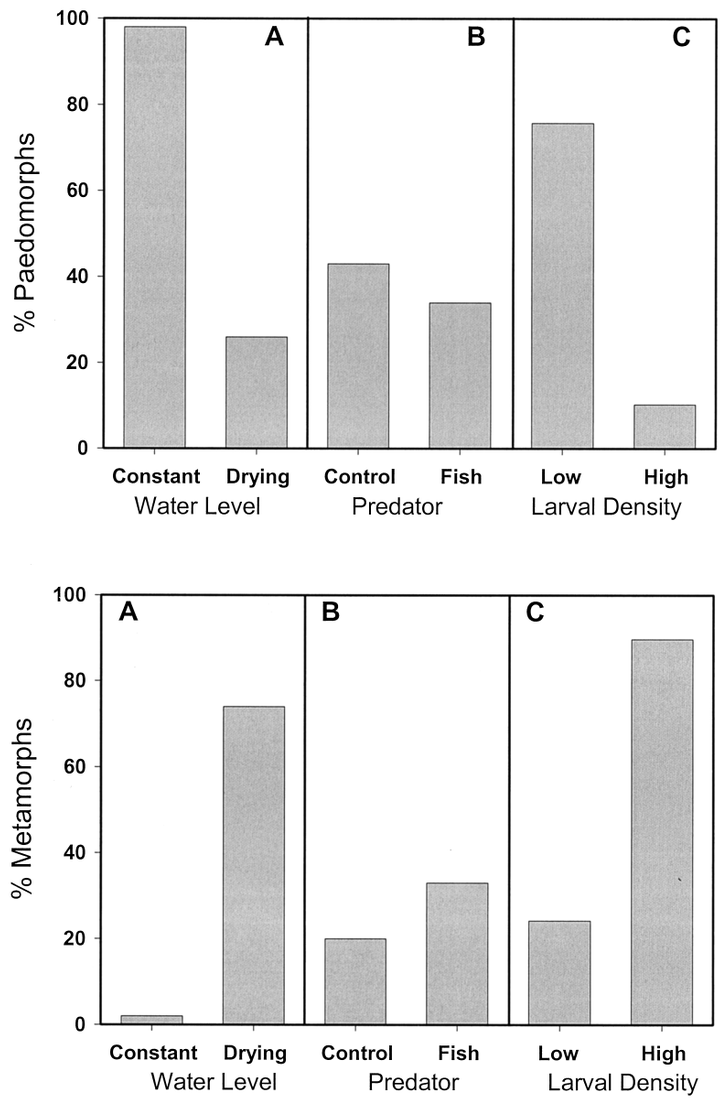

FIGURE 2 Percentages of paedomorphs (top graph) and metamorphs (bottom graph) observed in the facultatively paedomorphic salamanders (A–B) *Ambystoma talpoideum* and (C) *Notophthalamus viridescens dorsalis* exposed to different environmental conditions. Larvae were reared under conditions of (A) constant or decreasing (drying) water level, (B) presence or absence of a fish predator, or (C) high or low population density. Data modified from (A) Semlitsch (1987a); (B) Jackson and Semlitsch (1993); (C) Harris (1987).

earlier than migrating metamorphs for any given breeding season. Paedomorphs are capable of courtship, sperm transfer, insemination, and oviposition prior to the arrival of terrestrial adults migrating to the breeding pond (Scott, 1993; Krenz and Sever, 1995). Because competition and other density-dependent factors influence growth and survival in many amphibian species (Harris, 1987; Semlitsch, 1987b; Taylor

and Scott, 1997), early reproduction by paedomorphic adults may allow their offspring time to grow prior to the hatching of larvae from terrestrial adults. Because of the earlier growth opportunities, larvae from paedomorphic adults may have a competitive advantage due to their larger size compared with larvae of terrestrial metamorphic adults. Early growth and survival benefits can later translate into enhanced adult performance and thus greater fitness (Semlitsch *et al.,* 1988). Paedomorphs retain the ability to undergo metamorphosis. However, this transition typically only occurs in the spring after the breeding season (Semlitsch, 1985; Whiteman, 1994).

The paedomorphic advantage model predicts metamorphosis is maintained in a population by selection acting primarily during the occasional years of unfavorable aquatic conditions. Higher proportions of metamorphs are generated during aquatic conditions such as low water levels (Semlitsch, 1987a), high conspecific density (Harris, 1987), and the presence of a fish predator (Jackson and Semlitsch, 1993) (Fig. 2). The transition to a terrestrial habitat not only allows larvae to escape deteriorating aquatic conditions, but also permits metamorphic adults to colonize newly formed pools during subsequent breeding seasons. Such pools may be free from predators and yield higher larval growth rates (Whiteman *et al.,* 1996).

Larvae receive input from their environment and choose a life history strategy that has the highest relative fitness for the prevailing ecological conditions (i.e., they exhibit developmental plasticity). The proximate mechanisms by which facultative paedomorphic salamanders select a particular life history trajectory are the subject of Section IV.

III. ENDOCRINOLOGY OF METAMORPHOSIS

A. Overview

Hormones orchestrate the diverse morphological and physiological changes that occur during metamorphosis (see Fig. 3). Gudernatch (1912) first showed **Fig. 3** 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 Barger, 1927), and referred to as thyroid hormone (TH), is now known to be the primary hormone controlling amphibian metamorphosis. Although hormones produced by the anterior pituitary gland and the interrenal glands (the amphibian homologs of the mammalian adrenal cortex) can influence the rate of metamorphosis, exogenous

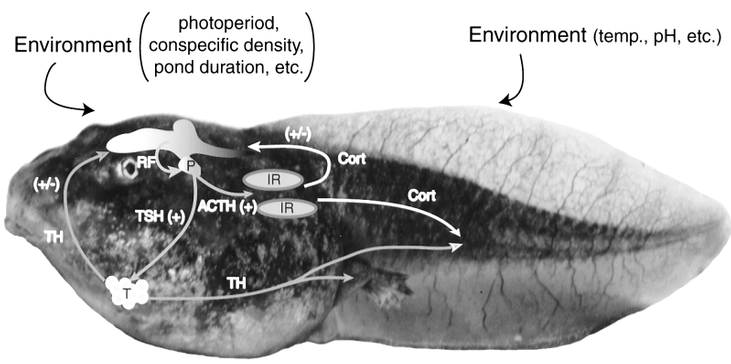

FIGURE 3 Endocrine systems controlling tadpole metamorphosis. ACTH, adrenocorticotropic hormone; Cort, corticoids; IR, interrenal gland; P, pituitary gland; RF, releasing factor; T, thyroid gland; TH, thyroid hormone; TSH, thyroid-stimulating hormone. Pluses indicate a stimulatory effect and minuses a negative feedback. In the case of TH and Cort effects on the brain, (+/−) indicates that these hormones promote differentiation of neurosecretory centers (and other brain regions) in addition to their negative feedback effects on neurohormone and pituitary hormone secretion.

TH alone can induce the entire suite of tissue transformations (see Kikuyama *et al.,* 1993; Shi, 1996). Furthermore, chemical or surgical thyroidectomy results in metamorphic stasis (Dodd and Dodd, 1976; Kikuyama *et al.,* 1993).

The work of William Etkin laid much of the foundation for our 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 among amphibian endocrinologists for describing the stages of anuran development: premetamorphosis, when the larvae grows but little or no morphological change occurs and plasma TH concentrations are low; prometamorphosis, when hind-limb growth accelerates and plasma TH concentration rises; and metamorphic climax, the final and most rapid phase of morphological change when thyroid activity is at its peak (see Dodd and Dodd, 1976; White and Nicoll, 1981; Table 2).

Table 2

The following section describes the cast of endocrine characters that interact to control metamorphosis. For each endocrine axis involved in metamorphosis we first examine its developmental schedule. This allows predictions of when the endocrine system is sufficiently developed to allow the animal to become competent to respond to the external environmental. We also examine the multiple levels at which the activity and functioning of each endocrine axis can be regulated. In Section V on integration, we address how the endocrine system determines the timing of metamorphosis and mediates environmental effects on amphibian development.

B. Thyroid Hormone

1. Role 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, death, differentiation, or migration. Target cells for TH are 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.

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 iodoproteins (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 to early prometamorphosis (Nieuwkoop and Faber, 1956; Saxén *et al.,* 1957a,b; Kaye, 1960; Dodd and Dodd, 1976; Regard *et al.,* 1978). Multiple measures of thyroid activity, including radioiodine uptake, gland ultrastructure, and plasma concentrations or tissue content of THs, show that thyroid activity increases markedly during prometamorphosis (Table 2), peaks at metamorphic climax, and declines thereafter to reach an adult level of activity (Kaye, 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 (see 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 (T_4; the primary product of the thyroid gland) and 3,5,3′-triiodothyronine (T_3; derived from T_4 by monodeiodination in target tissues; see Fig. 4) concentrations during metamorphosis. These studies confirmed earlier studies and the predictions of Etkin by demonstrating low to nondetectable plasma TH concentrations during premetamorphosis, increasing concentrations during prometamorphosis, and a dramatic peak at

Fig. 4

TABLE 2
Comparison of Three Staging Tables for Postembryonic Feeding Stages of Anuran Larvae[a]

N-F staging[b] for X. laevis	*Major common diagnostic features and morphological changes*	*T-K staging[c]*	*Gosner staging[d]*	*Etkin terminology[e]*
1–45	Nonfeeding stages (comparable to Shumway stages[f] 1–24)		1–25	
46		I	26	Premetamorphosis
47–48	Feeding begins	II	27	
49–50		III	28	
51		IV	29	
		V	30	
52	Foot-paddle stages			
		VI	31	
53		VII	32	
		VIII	33	
54		IX	34	
		X	35	
55	Hind-limb stages	XI	36	Prometamorphosis
		XII	37	
56		XIII	38	
57–58		XIV–XVI	39–40	
59	Tadpole reaches maximum length	XVII	40	
60		XVIII	41	
		XIX		
61		XX		
62	Rapid tail resorption begins, front limbs erupt[g]	XXI	42	Climax
63		XXII	43	
64		XXIII	44	
65	Stump of tail remains	XXIV	45	
66	Tail completely resorbed, juvenile frog	XXV	46	

[a] 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 *X. laevis* and the staging of *R. 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).

[b] Nieuwkoop and Faber (1956).

[c] Taylor and Kollros (1946).

[d] Gosner (1960).

[e] Etkin (1968).

[f] Shumway (1940).

[g] The front limbs erupt in *X. laevis* at stage 58 and continue to grow and develop through metamorphic climax. In other amphibians, such as ranids or pelobatids (e.g., *Scaphiopus*), the front limbs develop internally and then erupt at metamorphic climax.

metamorphic climax (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.,* 1991b).

Because of the difficulty of obtaining blood from small tadpoles for analysis by RIA, only those species with tadpoles large enough to obtain a serum sample were analyzed. Thus, most blood measurements

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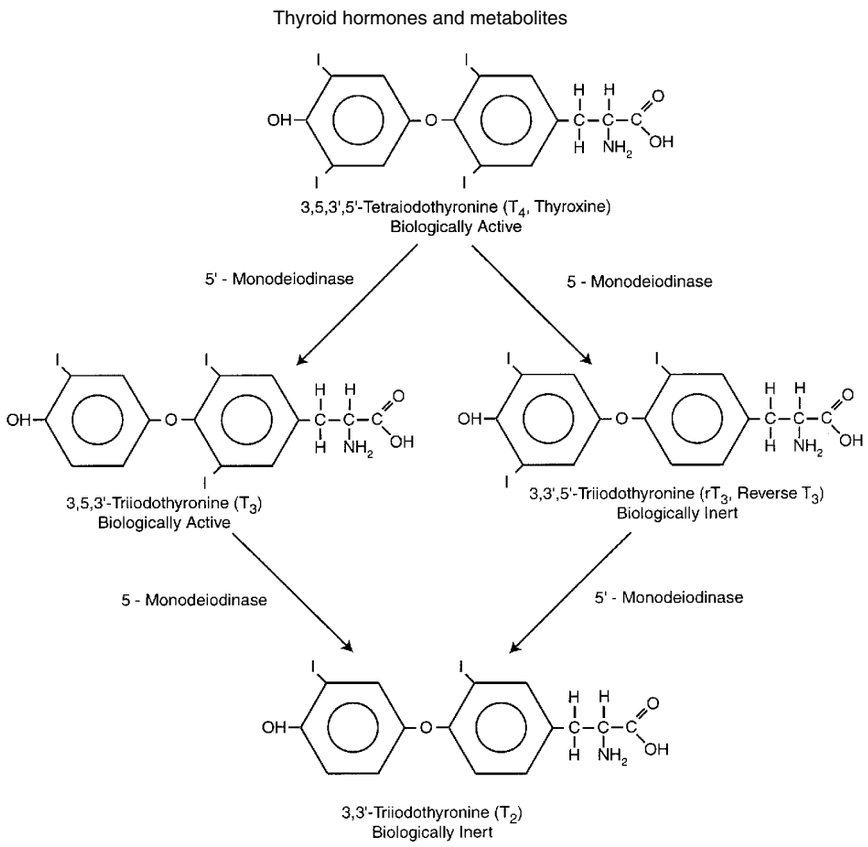

FIGURE 4 Thyroid hormone structure and metabolism. Arrows indicate deiodination by tissue monodeiodinases, resulting in bioactivation or bioinactivation of the substrate.

have been done on ranid species (e.g., *Rana catesbeiana* and *Rana clamitans*); however, Leloup and Buscaglia (1977) and Tata *et al.* (1993) have measured THs in plasma pools of *X. laevis* (see also Buscaglia *et al.*, 1985, for measures of plasma T_3 and T_4 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.,* 1991b), *Spea hammondii* (Denver, 1993, 1997a, 1998), *X. laevis* (R. J. Denver, unpublished data), *Bufo marinus* (Weber *et al.,* 1994). The peak in whole-body T_3 and T_4 coincides with peak uptake of ^{131}I in *Bufo japonicus* (Niinuma *et al.*, 1991b). 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.

3. Control of Thyroid Hormone Secretion, Metabolism, and Transport

a) Pituitary Control The increase in thyroid gland growth and biosynthetic activity during prometamorphosis is dependent on the pituitary hormone thyrotropin (thyroid-stimulating hormone, TSH). The development of the thyroid gland is arrested in hypophysectomized tadpoles, resulting in the failure to metamorphose (Regard and Mauchamp, 1971, 1973; Dodd and Dodd, 1976). This condition can be reversed by injecting TSH (Regard and Mauchamp, 1971, 1973). It is likely that the early development of the thyroid gland does not depend on TSH because its development

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occurs before immunoreactive TSH cells are present in the anterior pituitary, which occurs at NF stage 42 in *X. 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.

Although functional thyroid follicles are present at stages that precede the prometamorphic rise in TH production, the rate of hormone synthesis is coordinate with the development of the pituitary gland and the production of TSH (Kaye, 1960; Dodd and Dodd, 1976; Buckbinder and Brown,1993; Denver, 1996). The amphibian thyroid gland develops sensitivity to TSH during late embryogenesis (just prior to hatching), as can be demonstrated by the increased radioiodine uptake by thyroids following TSH injection (see Kaye, 1960). There have been no direct measures of circulating TSH (by RIA) in amphibians. However, evidence for an increase in circulating TSH at the early limbbud stage (T-K stage III) in *R. pipiens* tadpoles was provided by Kaye (1961).

Thyrotropin is composed of two subunits, α and β, that are derived from two separate genes. The α subunit is common among the glycoprotein hormones (i.e., the gonadotropins (GtHs), luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and TSH); whereas, the β subunit confers hormonal specificity on the molecule (Pierce and Parsons, 1981). The cDNAs for TSHβ subunit have now been isolated from three amphibian species—two anurans, *X. laevis* (Buckbinder and Brown, 1993) and *R. catesbeiana* (Okada *et al.,* 2000), and one urodele, *Hynobius retardatus* (Kanki and Wakahara, 2000; partial cDNA). Deduced amino acid sequences of the amphibian TSHβ subunits show that they exhibit 40–73% sequence similarity to known vertebrate TSHβ molecules. Northern blot analysis of the developmental expression of pituitary α glycoprotein subunit and TSHβ subunit mRNAs in *X. laevis* tadpoles did not detect the expression of these genes at NF stage 52 but showed a dramatic increase in expression by stage 57 (stages between 52 and 57 were not analyzed; Buckbinder and Brown, 1993). A similar developmental schedule of TSHβ mRNA expression was demonstrated in the bullfrog (Okada *et al.,* 2000). 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.

b) Thyroid Hormone Conversion The major product of the amphibian thyroid gland is T_4, with minor amounts of T_3 produced (see Rosenkilde, 1978; Buscaglia *et al.,* 1985; Fig. 4). The result is that the plasma T_4 concentration tends to be an order of magnitude greater than T_3 (Regard *et al.,* 1978; Larras-Regard *et al.,* 1981). The only case in which this relationship may not hold is for *X. laevis,* in which the reported plasma T_3:T_4 ratio is very similar and may even exceed 1 at metamorphic climax (Leloup and Buscaglia, 1977; Buscaglia *et al.,* 1985). Measures of the tissue content of T_4 and T_3 in various species show that the two hormones are present in roughly similar amounts (Niinuma *et al.,* 1991b; Weber *et al.,* 1994; Denver, 1997a, 1998). 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 T_3:T_4 ratio in tissues compared with T_3:T_4 ratios in blood reflects high tissue 5′-monodeiodinase activity.

Tissue monodeiodinases convert T_4, the product of the thyroid gland, to T_3 by removing one iodine atom at the 5′-position (see Fig. 4). T_3 is often referred to as the biologically active form of TH because the TH receptors (TRs) possess 10 times greater affinity for T_3 than for T_4 (see Leonard and Visser, 1986; Oppenheimer *et al.,* 1995). Similarly, T_3 exhibits 3–10 times greater biological activity than T_4 in amphibia as it does in other vertebrates (Wahlborg *et al.,* 1964; Lindsay *et al.,* 1967; Rosenkilde, 1978; Frieden, 1981; White and Nicoll, 1981). Thus, data support the view that, although T_4 is the primary product of the thyroid gland, T_3 derived from conversion in the target tissues is the biologically active form of the hormone. T_4 can also be inactivated by conversion to reverse T_3 (3,3′,5′-triiodothyronine; rT_3) and diiodothyronine (T_2); neither compound binds to the TRs. Similarly, T_3 can be inactivated by deiodination (Fig. 4).

The tissue deiodinases catalyze two basic reactions—a 5′-monodeiodination (outer ring) that results in bioactivation and a 5-monodeiodination (inner ring) that results in the bioinactivation of the substrate, T_4, or T_3 (Fig. 4). Three types of vertebrate deiodinases

have been described that differ in their substrate specificity, kinetics, and sensitivity to inhibitors. Thus, the isozymes were originally identified by operational definitions based on their biochemical and pharmacological characteristics and not as specific polypeptides. However, cloning of cDNAs for subunits of each of these enzymes allows the assignment of biochemical attributes to specific proteins (see St. Germain, 1994).

Tadpoles possess both 5- and 5′-deiodinase activities; although, the enzymes exhibit primarily type II and type III activities, with no evidence for an enzyme with type I characteristics (Becker *et al.,* 1997). Complementary DNAs for two enzymes, presumably corresponding to these two different activities, have been cloned in *R. catesbeiana;* an amphibian type III enzyme was first cloned in *X. laevis* (St. Germain *et al.,* 1994). In *R. catesbeiana,* these two enzymes exhibit tissue-specific and developmental stage-specific expression patterns. For simplicity, in the following discussion we abbreviate the type II enzyme as D2 and the type III enzyme as D3.

During metamorphosis, coincident with rising plasma titers of T_3 and T_4, there is an increase in both D2 and D3 activities in target tissues (Buscaglia *et al.,* 1985; Galton, 1991; Brown *et al.,* 1996; Becker *et al.,* 1997; Kawahara *et al.,* 1999). In bullfrog tadpoles the D2 and D3 enzymes exhibit differential tissue expression. For example, D2 enzyme activity (and mRNA) is expressed in the tail, intestine, hind limb, forelimb, eye, and skin, but no D2 could be detected in the liver or kidney of bullfrog tadpoles at any stage (Galton, 1988; Galton and Hiebert, 1988; Becker *et al.,* 1997). This finding contrasts sharply with many other vertebrates in which both the liver and kidney possess high 5′-deiodinase activities, and both organs are thought to be the primary sources of circulating T_3 (St. Germain and Galton, 1997). By contrast with D2, D3 enzyme activity (and mRNA) is expressed in liver and kidney as well as the tail, intestine, hind limb, forelimb, eye, skin (*R. catesbeiana,* Becker *et al.,* 1997; *X. laevis,* Wang and Brown, 1993; Brown *et al.,* 1996), and brain (*X. laevis* head, Brown *et al.,* 1996; brain, Denver *et al.,* 1997).

In tissues in which both enzymes are expressed, D2 and D3 exhibit comparable ontogenetic expression profiles (Becker *et al.,* 1997). In the bullfrog tadpole, the expression patterns of each of these genes correlate well with the schedule of metamorphic changes in particular organs. For example, D2 activity is highest in hind limbs during prometamorphosis, at which time the limbs are differentiating, and declines at metamorphic climax. In the tail, which is the last organ to undergo metamorphic transformation (resorption), D2 activity is very low until metamorphic climax. The D3 activity exhibited similar ontogenetic profiles (Becker *et al.,* 1997). These findings led Becker *et al.* (1997) to hypothesize that the coexpression of the two enzymes during metamorphosis generates a push–pull mechanism, thereby providing for tight control of intracellular T_3 concentrations in tissues at times of maximum metamorphic changes. However, although these findings in the bullfrog were partially corroborated in *X. laevis* for D3 mRNA expression, species differences were also evident (Kawahara *et al.,* 1999). D3 mRNA in *X. laevis* showed similar ontogenetic profiles to *R. catesbeiana* in the tail, intestine, and liver, but the hind limb and kidney showed patterns of expression that were directly opposite. D2 mRNA expression in *X. laevis* has not been analyzed. The meaning of such species differences in expression patterns is unknown, but must be understood in order to derive general principles regarding the roles that the deiodinases play in regulating tissue responsiveness to TH during metamorphosis.

The regulation of deiodinase gene expression is poorly understood. Conflicting results for the regulation of D2 activity have been published. Buscaglia *et al.* (1985) reported that in *X. laevis* treated with the goitrogen perchlorate D2 activity remained at low premetamorphic levels. Replacement with T_3 or T_4 in these animals induced D2 activity, suggesting that TH positively regulates 5′-deiodination. By contrast, Becker *et al.* (1997) reported that in bullfrog tadpoles treated with the goitrogen methimazole D2 activity was elevated and replacement with T_4, but not T_3, down-regulated this activity. D3 enzyme activity and mRNA are clearly up-regulated by T_3. The cDNA for the *X. laevis* D3 gene was twice isolated as a T_3-regulated gene in differential screens of the tail and brain (Wang and Brown, 1993; Denver *et al.,* 1997). Response kinetics and the resistance of up-regulation of the mRNA to protein synthesis inhibition suggest that it is a direct T_3 response gene. This gene is up-regulated in the tail, brain, intestine and hind limb, but is down-regulated in the liver (Wang and Brown, 1993; Denver *et al.,* 1997; Kawahara *et al.,* 1999). This pattern of T_3 responsiveness fits the ontogenetic expression profiles for the gene

when it is up-regulated during late prometamorphosis to metamorphic climax in each of the tissues in which it responds positively to the hormone but down-regulated in the liver (Kawahara *et al.,* 1999). Clearly, the roles of THs and other physiological and environmental factors in the regulation of deiodinase gene expression and enzyme activity require further study.

What is the evidence for a physiological role for tissue deiodinases in the control of metamorphosis? Several investigators have treated tadpoles with iopanoic acid (IOP), which blocks D2 and D3 activities in tadpoles (Buscaglia *et al.,* 1985; Galton, 1989; Becker *et al.,* 1997). The hypothesis tested was: If conversion of T_4 to T_3 is important for the metamorphic process, then IOP should block metamorphosis. As predicted, treatment with IOP inhibited metamorphosis, and this blockade could be overcome by replacement with T_3 but not T_4 (Galton, 1989; Becker *et al.,* 1997). These findings support the view that T_3 is the biologically active hormone and its generation from T_4 is essential to metamorphosis. Similarly, the importance of the degradation of THs to the coordination of metamorphic transformations is supported by studies with transgenic frogs. The overexpression of a D3 green-fluorescent-protein (GFP) fusion protein in transgenic *X. laevis* resulted in metamorphic stasis and resistance to exogenous TH (Huang *et al.,* 1999). At a finer level, D3 has been implicated in the modulation of T_3-dependent development of the visual system in tadpoles (Marsh-Armstrong *et al.,* 1999). Taken together, the data point to a central role for tissue deiodinases in modulating tissue responsiveness to T_3 through their exertion of tight control over intracellular concentrations of the hormone.

c) Thyroid Hormone Transport in Blood Once synthesized, T_4 diffuses out of thyroid follicular cells and into the bloodstream, where it becomes reversibly bound to plasma proteins. The plasma proteins serve to transport the hormone from the site of production to its target tissues. Several vertebrate plasma-binding proteins that bind T_4 and T_3 with varying affinities have been identified. Thyroxine-binding globulin (TBG) is found only in large eutherian mammals, and it binds T_4 with high affinity and low capacity (Power *et al.,* 2000). Transthyretin (TTR; also known as prealbumin) is found in all vertebrates and it binds T_4 with moderate affinity and intermediate capacity. Both TBG and TTRs can also bind T_3, although in most cases with 10 times lower affinity than T_4 (Power *et al.,* 2000); however, the situation in amphibia is the reverse—see later). The two primary sites for TTR expression in vertebrates are the liver and the choroid plexus (although it is expressed at other sites; see Power *et al.,* 2000). In most mammals TTR is expressed in both tissues, in reptiles it appears to be expressed only in the choroid plexus, and in teleosts and amphibians it is expressed primarily in the liver (see Power *et al.,* 2000; although see Funkenstein *et al.,* 1999, for TTR expression in the skin and other tissues of the teleost fish, *Sparus aurata*). 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 T_3 and 9-*cis*-retinoic acid (which is a metabolite of all-*trans*-retinol) serve as ligands for the TR–retinoid-X receptor (TR-RXR) heterocomplex. Evidence supports the hypothesis that the TR-RXR heterodimer is the active complex that binds to promoters of TH target genes and activates transcription in the presence of TH (see later). Serum albumin also binds T_3 and T_4 in many species with low affinity and high capacity. Power *et al.* (2000) suggest that albumin might be the principal T_4-binding protein in amphibia.

By contrast with other tetrapods, but similar to teleost fishes, amphibian TTRs exhibit much greater affinity for T_3 than for T_4 (Yamauchi *et al.,* 1993, 1998, 1999, 2000). The functional significance of the apparent evolutionary transformation of TTR from a T_3-binding to a T_4-binding protein is not known (see Power *et al.,* 2000). In *R. catesbeiana,* the binding affinity of TTR for T_3 is 100–360 times greater than for T_4 (Yamauchi *et al.,* 1993, 2000). Bullfrog TTR exhibits low nanomolar affinity for T_3—using TTR purified from plasma, 0.67 nM (Yamauchi *et al.,* 1993); using whole plasma or recombinant TTR, 8–9 nM (Yamauchi *et al.,* 2000). By contrast, the affinity of recombinant *X. laevis* TTR for T_3 is much lower than the bullfrog protein (550 nM; Yamauchi *et al.,* 2000). However, a similar relationship between the affinities of TTR for T_3 and T_4 exists in *X. laevis* (affinity for T_4, 13 μM; Yamauchi *et al.,* 2000). Circulating TTR protein is present in bullfrog and *X. laevis* tadpoles during premetamorphosis and prometamorphosis, but declines at metamorphic climax (Yamauchi *et al.,* 1998, 2000).

What might be the functional significance of the developmental expression pattern of TTR in tadpoles? TTR expression is high during prometamorphosis when thyroid activity is increasing (see previous discussion) and plasma T_4 and T_3 concentrations are rising. Based on the free hormone hypothesis (Mendel, 1989; Ekins, 1990), we predict that TTR at this stage of development reduces the free fraction of hormone in the blood and thus limits the availability of the hormone to target tissues. On the other hand, TTR serves as a sink for the hormone in the blood, thus maintaining increasing plasma concentrations of THs before thyroid gland activity accelerates in response to rising titers of plasma TSH. At metamorphic climax, when plasma T_3 and T_4 concentrations are maximal, TTR concentration in the blood declines. The continued rise in plasma TH concentrations (without a high-affinity plasma protein binder to slow hormone clearance) probably results in an increased free hormone fraction (at least for T_3) in the blood. At the same time, the rate of clearance of T_3 from the circulation probably increases. However, because the thyroid synthetic rate is so high at metamorphic climax, total T_3 concentrations continue to rise. Thus, we predict that not only does the hormone production rate increase at metamorphic climax, but also the proportional availability of T_3 to the target tissues. To our knowledge T_3 or T_4 clearance rates have not been calculated in tadpoles at different stages of development. Based on TTR expression profiles we predict that clearance rates are lower during prometamorphosis than during premetamorphosis or metamorphic climax. Furthermore, given the lower affinity of TTR for T_4 compared with T_3, we predict that the clearance rate for T_4 is higher than T_3.

d) Cellular Uptake of Thyroid Hormone It was once thought that, because of their lipophilicity, THs entered cells by simple diffusion across plasma membranes. However, the highly polar nature of the alanine side chain precludes free membrane passage of the iodothyronines (Friesema *et al.,* 1999). It is clear that THs can be actively taken up by cells via plasma membrane transporters (Hennemann *et al.,* 1998). The saturable, carrier-mediated uptake of THs has been demonstrated in rat liver cells (Rao *et al.,* 1976; Krenning *et al.,* 1981), cultured fibroblasts (Cheng *et al.,* 1980), human and rat red blood cells (RBCs; Docter *et al.,* 1982; Zhou *et al.,* 1992), rat thymus cells, and tadpole RBCs (Galton *et al.,* 1986; Yamauchi *et al.,* 1989).

Findings point to an important role for amino acid permeases in the uptake of THs by cells (see Ritchie *et al.,* 1999). The T_3-inducible gene IU12 from *X. laevis* intestine (Shi and Brown, 1993; Liang *et al.,* 1997) encodes a subunit of a heterodimeric amino acid permease complex (Torrents *et al.,* 1998). Findings by Ritchie and colleagues (1999) show that this permease complex efficiently transports T_3 and T_4 when expressed in the *Xenopus* oocyte expression system, but is inhibited by reverse T_3. The fact that the IU12 is a T_3-inducible gene suggests that it might play a role in mediating T_3 uptake by cells during tadpole metamorphosis (see Liang *et al.,* 1997). Other TH transporters that have been identified include organic anion transporters such as Ntcp and oatp1-3 (Abe *et al.,* 1998; Friesema *et al.,* 1999). The possibility for specific receptors for TTR also has been demonstrated, although this means of hormone uptake requires further investigation (see Divino and Schussler, 1990; Schussler, 2000).

e) Cytosolic Thyroid Hormone Binding Proteins Upon entering cells, and before binding to nuclear receptors (see later), THs encounter a series of intracellular binding proteins. These cytoplasmic TH binding proteins (CTHBPs) are represented by several classes of multifunctional proteins. These proteins represent a variety of enzymatic activities in the cell. For example, two genes were cloned in *X. laevis* that are CTHBPs. One is a cytosolic aldehyde dehydrogenase that catalyzes the formation of retinoic acid (an important developmental signaling molecule that signals via nuclear receptors; see later; Yamauchi and Tata, 1994), and the other is homologous to mammalian M2 pyruvate kinase (Shi *et al.,* 1994). Protein disulfide isomerase (PDI) and related proteins catalyze the formation of disulfide bonds in and between proteins, and human PDI possesses a high-affinity binding site for TH (Cheng *et al.,* 1987; Yamauchi *et al.,* 1987). We cloned a cDNA encoding a PDI-like protein as a T_3-responsive gene in the *X. laevis* brain (Denver *et al.,* 1997).

It has been suggested that the functional significance of hormone binding to these CTHBPs is to serve to transport THs in the cytoplasm to the nucleus where the TRs are located. Alternatively, they could serve as

chelators to limit the cellular free-TH concentration or act as buffer proteins in the maintenance of intracellular levels of TH (see Shi, 2000). However, in considering a role for these proteins in TH transport, the possibility that TH might serve a regulatory role for the enzymatic activities of these proteins should not be overlooked. As an example, the human M2 pyruvate kinase functions as a kinase in its tetrameric form, but only binds TH in its monomeric form. The binding of TH results in a shift toward the monomeric form and thus the inhibition of the kinase activity (Ashizawa and Cheng, 1992). Thus we predict that TH serves to inhibit this enzymatic pathway.

4. Mechanisms of Thyroid Hormone Action: Thyroid Hormone Receptors

Tadpoles become competent to respond to exogenous TH at the time of hatching (Tata, 1968). This establishment of competence to respond to the hormone probably depends on the expression of TRs (see Shi *et al.,* 1996). TRs are ligand-activated transcription factors that belong to the steroid hormone receptor superfamily (Mangelsdorf and Evans, 1995). There are two TR genes, termed α and β, in all vertebrates (Lazar, 1993). Owing to its pseudotetraploidy, *X. laevis* possesses four TR genes, two α and two β (Brooks *et al.,* 1989; Yaoita *et al.,* 1990). The two *X. laevis* TRα genes each appears to give rise to a single unique protein, whereas alternative mRNA splicing of TRβ transcripts can give rise to two different receptor isoforms for each TRβ gene (Yaoita *et al.,* 1990; Shi, 2000).

The TRα 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; Yaoita and Brown, 1990; Banker *et al.,* 1991; Kawahara *et al.,* 1991). It has been hypothesized that the early expression of TRα establishes the hormone responsiveness of tadpole tissues (see Baker and Tata, 1990; Shi *et al.,* 1996). TRβ mRNA is not detected until early prometamorphosis, but its expression increases during prometamorphosis in parallel with TH synthesis (Yaoita and Brown, 1990; Kawahara *et al.,* 1991; Baker and Tata, 1992; Kanamori and Brown, 1992). Several studies have shown that the TR genes are up-regulated by T_3 in *X. laevis* and *R. catesbeiana* (Yaoita *et al.,* 1990; Kawahara *et al.,* 1991; Schneider and Galton, 1991; Helbing *et al.,* 1992), a phenomenon termed autoinduction (see Tata *et al.,* 1993; Davey *et al.,* 1994; Rabelo and Tata, 1997). A thyroid response element (TRE), 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).

The specific functions for the different receptors in amphibia are unknown. The results of gene-targeting experiments in mice point to a network of specific and common TR pathways, but have failed to provide a clear picture of the roles for these different receptors (Forrest and Vennstrom, 2000). There is evidence in mammals that the TRs possess different functional characteristics (Zhu *et al.,* 1999) and can mediate different cellular responses to T_3 (Lebel *et al.,* 1993), presumably by regulating different sets of genes (Guissouma *et al.,* 1998; Sandhofer *et al.,* 1998; Denver *et al.,* 1999). Studies addressing specific functions for the different TRs have not been done in amphibians.

TRs 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 make up a TRE (Williams and Brent, 1995). These TREs can be located in the promoter, in 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) (see 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 activational actions of the complex (Shi, 2000; Wu and Koenig, 2000). The TR and RXR genes exhibit more or less coordinated regulation during metamorphosis, and this coordination may be essential to the timing of tissue-specific changes (Wong and Shi, 1995).

Hormone binding to the TR-RXR complex induces gene expression in target tissues. A detailed discussion of the characteristics of the gene-regulation cascades and the functions of the gene products induced in different tissues during metamorphosis is beyond the scope of this chapter. The reader is referred to Shi (2000) for a thorough treatment of this topic.

C. Corticoids

Although TH is the primary morphogen controlling metamorphosis, corticoids may synergize with TH to accelerate metamorphosis (Kikuyama *et al.,* 1993). Corticoids are the primary vertebrate stress hormones and are produced in response to a variety of environmental signals (Selye, 1976). The production of corticoids changes with development and probably reflects the functional maturation of the hypothalamic-hypophyseal-interrenal axis.

1. Roles of Corticoids in Amphibian Growth and Development

Corticoids (also referred to as corticosteroids) may influence growth and development in larval anurans, but their influence may be more complex than that of TH. Exogenous corticoids can either accelerate or decelerate metamorphosis, depending on the animal's developmental stage and TH status. Studies using relatively large doses of exogenous corticoids have shown that these hormones inhibit forelimb emergence when administered during premetamorphosis (Frieden and Naile, 1955; Kobayashi, 1958; Gray and Janssens, 1990; Hayes *et al.,* 1993; Wright *et al.,* 1994; Hayes, 1995). The effects of exogenous corticoids on tadpole growth are more straightforward than their developmental effects. The administration of various corticoid doses to both pre- and prometamorphic tadpoles inhibits growth (Hayes and Licht, 1993; Wright *et al.,* 1994; Hayes, 1995; K. A. Glennemeier and R. d. Denver, submitted).

Although exogenous corticoids when administered alone during premetamorphosis can inhibit growth and development, the hormones accelerate TH-induced metamorphosis in most species (Frieden and Naile, 1955; Kikuyama *et al.,* 1983, 1993; Gray and Janssens, 1990; Hayes, 1995). In one study, prometamorphic *Bufo boreas* tadpoles exposed to exogenous corticosterone alone also showed accelerated metamorphosis, probably due to synergy of the corticosterone with rising endogenous TH levels (Hayes *et al.,* 1993).

The studies in which tadpoles were treated with exogenous corticoids with or without TH suggest, but do not prove, a physiological role for endogenous corticoids in the regulation of tadpole development. Inhibitors of corticoid synthesis have been used to address the role of endogenous corticoids. Hayes and Wu (1995) found that a 33% reduction in corticosterone by treatment with metyrapone (an inhibitor of corticoid biosynthesis) slowed TH-induced acceleration of hind-limb development, but did not affect the rate of tail resorption (Hayes, 1995; Hayes and Wu, 1995). K. A. Glennemeier and R. d. Denver (submitted) found that a 50% reduction in whole-body corticosterone by treatment with metyrapone throughout prometamorphosis increased size at metamorphosis by more than 10%, but did not affect the rate of metamorphosis in *R. pipiens* tadpoles. More work is required to determine a potential role for endogenous corticoids in tadpole growth and development.

In summary, the dose of corticoid administered, the stage at which the hormone is given, and whether it is administered with TH determines the developmental effects of the steroid (K. A. Glennemeier and R. d. Denver, submitted). Whether these effects represent physiological actions remains to be determined. If these actions turn out to be physiologically relevant, then we predict that increased corticoid biosynthesis (perhaps in response to a stressor) in premetamorphic tadpoles might retard growth and delay metamorphosis. Conversely, increased corticoids in prometamorphic tadpoles might retard growth but accelerate metamorphosis.

2. Hormones Produced by Amphibian Interrenal Glands

Corticosterone and aldosterone appear to be the major corticoids produced by the amphibian interrenal glands (Carstensen *et al.,* 1961; Macchi and Phillips, 1966). In many species there is an elevation in plasma concentrations of these hormones during metamorphic climax that is more or less synchronous with plasma TH increases (see later).

The interrenal gland is generally less active in early premetamorphic developmental stages and more active during prometamorphosis and metamorphic climax (see 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 in Dodd and Dodd, 1976; however, see later for contradictory evidence). Activity of the interrenal enzyme Δ^5-3β-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, *Ambystoma tigrinum*.

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

Few have analyzed the activity of the hypothalamic-pituitary-interrenal axis throughout metamorphosis at levels other than the interrenal gland. Carr and Norris (1990) reported low immunoreactive corticotropin-releasing hormone (CRH) in the median eminence and arginine vasotocin (AVT) in the preoptic nucleus of premetamorphic *R. catesbeiana* tadpoles, which increased dramatically by late prometamorphosis. Both CRH and arginine vasopressin (AVP)—AVT is the amphibian hormone—are potent stimulators of adrenocorticotropic hormone (ACTH) secretion by cultured adult frog pituitaries (Tonon *et al.,* 1986). Note also that CRH is a potent and potentially important regulator of TSH secretion in tadpoles (discussed later). To our knowledge, no direct measures of ACTH production over development have been reported in amphibia. However, the expression of the messenger RNA for the precursor of ACTH, proopiomelanocortin (POMC) in the anterior pituitary of bullfrog tadpoles is low during premetamorphosis, increases during prometamorphosis, and remains high during metamorphic climax (Aida *et al.,* 1999). Whether this mRNA expression pattern reflects the production and secretion of ACTH peptide is unknown.

The tadpole hypothalamic-hypophyseal-interrenal axis becomes functional during premetamorphosis. For example, the interrenal glands of premetamorphic tadpoles of *R. pipiens* and *X. laevis* respond to ACTH injections *in vivo* by increasing whole-body corticosterone content (Glennemeier and Denver, submitted). These experiments show that functional ACTH receptors are expressed before metamorphosis. The functionality of higher levels of the hypothalamic-hypophyseal-interrenal axis in premetamorphic animals is shown by their ability to mount a corticosterone response (increased whole-body corticosterone content) following exposure to an artificial stressor (handling and shaking stress in the laboratory; K. A. Glennemeier and R. J. Denver, submitted). Thus, there is the potential for environmental stressors to cause elevations in endogenous corticoid biosynthesis during premetamorphosis. Such early activation of the hypothalamic-hypophyseal-interrenal axis could result in growth retardation and metamorphic inhibition (see previous discussion).

3. Control of Corticoid Production and Transport

The major regulator of interrenal corticoid production is the pituitary hormone ACTH (Kikuyama *et al.,* 1993). Injections of ACTH increased serum corticoids and accelerated T_4-induced metamorphosis in several amphibian species (see White and Nicoll, 1981; Kikuyama *et al.,* 1993). The secretion of ACTH may be controlled by the neurohormones CRH and AVT (see previous discussion).

Corticoids, being lipophilic, are transported in blood bound to plasma proteins. Corticoid-binding globulin (CBG) is the primary plasma protein to which

corticoids bind in mammals, although albumin also plays a transport role (Hammond, 1990; Rosner, 1990). The 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 nor is there anything known of the role that this protein might play in maintaining corticoid balance in frogs or tadpoles.

4. Mechanisms of Corticoid Action

Corticoids, like 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 TRs (see previous discussion). Corticoid 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). The binding of the hormone results in the dissociation of the foldosome complex and translocation of the receptor to the nucleus (Pratt and Toft, 1997). Vertebrates possess two distinct corticoid receptors (designated glucocorticoid and mineralocorticoid) and both types have been isolated in *X. laevis* (Gao *et al.,* 1994a,b; Csikos *et al.,* 1995).

How might corticoids act to inhibit growth and development? In mammals, corticoids are known to produce growth inhibition through actions at multiple levels. At the organismal physiological level, corticoids mobilize stored fuels during increased metabolic demand—for example, fight-or-flight response, exercise, or fasting (see Sapolsky *et al.,* 2000). The chronic elevation of plasma corticoid concentrations promotes protein catabolism and muscle wasting. Corticoids are known to down-regulate growth hormone (GH) biosynthesis in the anterior pituitary gland of mammals (see Harvey *et al.,* 1995).

Corticoids may enhance the developmental actions of TH by several mechanisms. Corticoids have been shown to increase maximal nuclear binding capacity for T_3 in a dose-dependent manner and thus alter tissue responsiveness (Niki *et al.,* 1981; Suzuki and Kikuyama, 1983; Kikuyama *et al.,* 1993). Our studies (Denver *et al.,* submitted) have found that corticosterone up-regulates TRα and TRβ mRNAs in *X. laevis* tail cultures. Corticosterone may also increase 5′-deiodinase activity, thereby increasing the availability of T_3 at peripheral tissues (Galton, 1990).

D. Prolactin and Growth Hormone

The pituitary hormones GH (also called somatotropin) and prolactin (PRL; also called lactotropin) are simple polypeptides approximately 200 amino acids in length and are paralogous members of a multigene family. A key component of Etkin's (1968) model was that the stimulatory actions of TH on metamorphosis were counterbalanced by the inhibitory effects of the pituitary hormone PRL. Etkin proposed that PRL production would be high during larval life and then decline at metamorphic climax. This prediction was based largely on the inhibitory effects that preparations of mammalian PRLs had on metamorphosis when injected into tadpoles (see White and Nicoll, 1981). Based on the antimetamorphic actions of these mammalian PRL preparations, several investigators suggested that PRL exerted a juvenilizing action in amphibian larvae akin to that of juvenile hormone in insects (Bern *et al.,* 1967; Etkin and Gona, 1967).

The early studies that led to the development of the Etkin model have been extensively reviewed (see Dodd and Dodd, 1976; White and Nicoll, 1981; Kikuyama *et al.,* 1993; Denver, 1996; Kaltenbach, 1996). Studies using primarily mammalian preparations of GH or PRL suggested different roles for these hormones, with PRL enhancing larval growth and blocking the actions of TH on metamorphosis, and GH primarily stimulating postmetamorphic growth as it does in other vertebrates (see Denver, 1996). A role for GH in regulating body growth in amphibia as it does in other vertebrates (see Harvey *et al.,* 1995) has been borne out by numerous studies in which GH was injected into tadpoles or frogs (see White and Nicoll, 1981; Kikuyama *et al.,* 1993; Denver, 1996) and through the use of transgenic techniques in *X. laevis* (Huang and Brown, 2000a). A role for PRL in the stimulation of tadpole growth and the inhibition of metamorphosis has been questioned (see Huang and Brown, 2000b).

The early studies supported the view that treatment of tadpoles with PRL inhibits metamorphosis and stimulates larval growth. Most of these studies, done with

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mammalian PRL (and GH) preparations, showed that tadpole tissues have the capacity to respond to PRL-like or GH-like molecules; functional receptors are expressed in amphibian tissues that can transmit a signal that can both promote tadpole growth and block T_3-induced metamorphosis, probably by preventing the autoinduction of the TRs (see Tata *et al.,* 1993). Furthermore, studies with amphibian PRL preparations show that the homologous PRL has effects similar to the mammalian hormones (see Kikuyama *et al.,* 1993). Passive immunization studies with PRL antisera suggested a physiological role for endogenous PRL (see Kikuyama *et al.,* 1993; Denver, 1996).

But do these effects represent pharmacological actions of the exogenous hormones? The strongest argument against a role for PRL as a juvenilizing hormone in amphibians comes from expression analyses. Recall that Etkin (1968) proposed that larval growth and metamorphosis are controlled by a balance between TH and PRL and that the two should show an inverse relationship in their blood concentrations at metamorphic climax. The rise in circulating concentrations of TH during prometamorphosis and climax have been confirmed (see previous discussion). However, circulating concentrations of PRL and levels of pituitary PRL mRNA are low during premetamorphosis and also rise, more or less in parallel with TH, during late prometamorphosis and climax (Clemons and Nicoll, 1977; Yamamoto and Kikuyama, 1982; Takahashi *et al.,* 1990; Niinuma *et al.,* 1991a; 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 (see Buckbinder and Brown, 1993). Similarly, [^{125}I]-PRL binding to kidney membrane fractions was low in premetamorphic bullfrog tadpoles and increased during metamorphic climax (White and Nicoll, 1979). Huang and Brown (2000b) measured PRL receptor (PRL-R) mRNA by northern blotting in whole *X. laevis* tadpole and tail tissue and found increased expression at metamorphic climax. Taken together, these PRL and PRL-R expression analyses argue against the hypothesis that PRL plays a juvenilizing role in amphibian metamorphosis (see Buckbinder and Brown, 1993; Huang and Brown, 2000b). However, Kikuyama and colleagues (1993) have argued, based on their experiments with passive immunization with antiserum to bullfrog PRL, that low levels of PRL during the premetamorphic to early prometamorphic period might be sufficient to support larval growth and inhibit TH action.

Huang and Brown (2000a,b) used a transgenesis approach to address the question of the roles of GH and PRL in amphibian development. They created transgenic tadpoles of *X. laevis* that overexpressed *X. laevis* GH, *X. laevis* PRL, or ovine PRL. The expression of the transgenes was driven by the simian cytomegalovirus (sCMV) promoter; thus, all tissues expressed the transgenes (i.e., expression was not restricted to the pituitary gland where the hormones are normally expressed). They found that overexpression of GH had no effect on the timing of metamorphosis, but resulted in larger tadpoles and larger juvenile frogs, a finding that confirms earlier studies in frogs and other vertebrates that GH promotes growth (see Harvey *et al.,* 1995). The overexpression of *X. laevis* PRL (xPRL) or ovine PRL (oPRL) did not alter the timing of metamorphosis, but blocked tail resorption in some tadpoles. The overexpression of the mRNAs was confirmed by northern blotting; however, they were unable to detect the xPRL in serum of transgenic frogs by western blotting, but apparently were able to detect the oPRL. The authors concluded that their results disprove the hypothesis that PRL is a juvenile hormone in *X. laevis.* One caution in this interpretation is that the PRL was overexpressed in all tissues throughout the entire developmental period. Such stage-inappropriate overexpression of a hormone might result in compensatory changes in physiological systems; alternatively, the PRL-responsive cells could become desensitized by receptor internalization following chronic exposure to very high concentrations of the hormone, which is a common phenomenon in endocrine systems.

Whether or not PRL plays any role in larval growth or development, the rise in PRL biosynthesis at metamorphic climax suggests that the hormone might either modulate the rapid tissue transformations that occur at climax (e.g., provide a brake on TH action in concert with the up-regulation of the 5-monodeiodinase; see Denver, 1996) or perhaps play an important physiological role in the postmetamorphic frog (see Huang and Brown, 2000b).

E. Neuroendocrine Control of Amphibian Development

The vertebrate neuroendocrine system comprises the hypothalamus and the pituitary gland. The major pituitary hormones and their roles in amphibian development have already been described. The secretion of these pituitary hormones and thus the production of hormones by peripheral endocrine glands (e.g., the 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 hypophyseal portal vessels that deliver blood to the anterior pituitary gland (Fig. 3). The importance of hypothalamic control of metamorphosis has long been recognized (reviewed by Kikuyama *et al.,* 1993; Denver, 1996). The anterior pituitary gland controls both the thyroid gland and the interrenals by the production of TSH and ACTH, respectively.

Although environmental influences on the timing of metamorphosis can occur at the level of peripheral tissues (e.g., direct thermal effects and osmotic effects), much environmental information is gathered by neural sensory systems and integrated in 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.

1. Neurohormones and the Control of Pituitary Secretion

Early studies suggested that the pituitary hormones TSH and ACTH are primarily under stimulatory hypothalamic control in amphibians (reviewed by Denver, 1996). There have been far fewer studies done on the hypothalamic control of ACTH in amphibia than on TSH. The available data show that ACTH can be stimulated by CRH and AVT *in vitro* (Tonon *et al.,* 1986). However, whether CRH or AVT play important roles in controlling ACTH secretion *in vivo* in amphibia, as they do in mammals, has not been established.

2. Thyrotropin-Releasing Hormone (Pyro-glutamyl-histidyl-proline-amide)

The tripeptide pyro-glutamyl-histidyl-proline-amide was the first hypophysiotropic peptide to be isolated and have its structure determined (Reichlin, 1989). It was named thyrotropin-releasing hormone (TRH) for its ability to stimulate the release of TSH in mammals, where it appears to be the principal stimulator of TSH secretion (see Morley, 1981). However, its role as a TSH-releasing factor (TRF) in nonmammalian vertebrates is less certain. Although 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 (see Norris and Dent, 1989; Kikuyama *et al.,* 1993; Denver, 1996). However, TRH can elevate plasma TH concentrations when injected into adult frogs (Darras and Kuhn, 1982) and can stimulate the release of thyrotropic bioactivity in cultured pituitaries from adults of several frog species (Denver, 1988; Jacobs and Kuhn, 1992). The possibility that TRH plays a hypophysiotropic role in larval amphibians is uncertain. It appears that pituitary TSH cell responsiveness to TRH is regulated in a developmental-stage-specific manner (see Denver, 1988; Denver and Licht, 1989a). Future studies should address the regulation of TRH receptor expression in the amphibian pituitary to explain changes in the responsiveness of the gland to the tripeptide.

3. Corticotropin-Releasing Hormone Is a Thyrotropin-Releasing Factor

Studies have shown that the stress neurohormone CRH is a potent stimulator of the thyroid axis in larval amphibians and other nonmammalian vertebrates (reviewed by Denver, 1999). CRH is a 41-amino-acid polypeptide that was first isolated based on its ability to stimulate ACTH secretion in mammals (Vale *et al.,* 1981; Turnbull and Rivier, 1997). The regulation of ACTH secretion by CRH in mammals is considered to be its primary hypophysiotropic role (Vale *et al.,* 1997). In mammals, the actions of CRH peptides, in addition to their hypophysiotropic role, include control of appetite, behavioral responses to stress (arousal and escape), and modulation of immune responses, among others (Vale *et al.,* 1997).

In nonmammalian species, CRH (and related peptides, sauvagine and urotensin I) have been found to

be potent releasers of TSH. CRH stimulates the thyroid axis in a fish (salmon; Larsen *et al.,* 1998), several amphibians, reptiles, and a bird (reviewed by Denver, 1999). For example, CRH injections result in elevations in circulating TH concentrations in the frog *(Rana ridibunda),* the chick embryo Meeuwis *et al.,* 1989), and adult turtle (*Trachemys scripta*; R. J. Denver and L. E. Licht, unpublished data; see Kuhn *et al.,* 1998). Injections of CRH peptides also elevate whole-body TH content in tadpoles of several species (Gancedo *et al.,* 1992; Denver, 1993, 1997a). A direct action of CRH on TSH secretion by the pituitary gland is supported by tissue culture studies using pituitaries from representatives of each nonmammalian vertebrate class. Specific radioimmunoassays for TSH in the salmon (Larsen *et al.,* 1998) and turtle (Denver and Licht, 1989b, 1991) have verified that secretion of TSH protein is stimulated by CRH. In species in which a specific TSH radioimmunoassay is not yet available, the release of TSH was demonstrated by bioassay—for example, amphibians (Denver, 1988; Denver and Licht, 1989a; Jacobs and Kuhn, 1992)—or by a subtractive method using specific RIAs for the gonadotropin β subunits and the α subunit (chicken: Geris *et al.,* 1996; Kuhn *et al.,* 1998). Interestingly, although CRH is stimulatory to TSH secretion by cultured salmon pituitaries, TRH lacks activity in this regard (Larsen *et al.,* 1998). Matz and Hofeldt (1999) demonstrated that CRH-immunoreactive fibers terminate in regions that contain TSH-positive pituitary cells in Chinook salmon. They proposed that the contiguous localization of CRH-positive fibers and TSH cells supports a physiological role for CRH in mediating TSH release, which supports the findings of Larsen and colleagues (1998), who showed that CRH is a potent TSH-releasing factor in salmon.

Taken together, the data point to an important and perhaps primitive role for CRH in the regulation of both the thyroid and the interrenal (adrenal) axes. A role for CRH in influencing thyroid activity in tadpoles and thus regulating metamorphosis comes from studies from several labs in different species, which showed that injections of CRH-like peptides can accelerate metamorphosis. Injections of CRH and related peptides accelerated metamorphosis in the anurans *Rana perezi* (Gancedo *et al.,* 1992), *R. catesbeiana, Spea (Scaphiopus) hammondii* (Denver, 1993, 1997a), and *Bufo arenarum* (Miranda *et al.,* 2000) and in the salamander *Ambystoma tigrinum* (G. C. Boorse and R. J. Denver, submitted). CRH injections elevated whole-body TH content of *R. perezi* and *S. hammondii* tadpoles (Gancedo *et al.,* 1992; Denver, 1993). In *S. hammondii,* injections of synthetic *X. laevis* CRH (which is identical in primary structure to *S. hammondii* CRH; G. C. Boorse and R. J. Denver, unpublished) produced a dose-dependent increase in whole-body T_3, T_4, and corticosterone when measured 4 hours after injection (Denver, 1997a).

Passive immunization with CRH antiserum slowed spontaneous metamorphosis in *R. catesbeiana* tadpoles (Denver, 1993). Also, injections of the CRH receptor antagonist α-helical $CRH_{(9-41)}$ blocked simulated pond drying-induced metamorphosis in *S. hammondii* (Denver, 1997a). Furthermore, hypothalamic CRH peptide content was increased in spadefoot toad tadpoles that accelerated metamorphosis in response to simulated pond drying (Denver, 1997a). Taken together, these findings support a physiological role for CRH in controlling metamorphosis. Because CRH is a stress neurohormone we hypothesized that endogenous CRH participates in environmentally induced (stress-induced) metamorphosis (Denver, 1997b; see Section V).

All vertebrates studied possess at least two CRH receptor (CRHR) subtypes and a secreted CRH binding protein (CRH-BP) (Fig. 6). There is nothing known of the tissue distribution, developmental expression, or hormonal regulation of the CRHRs in amphibians. The CRH-BP has a high-affinity binding for CRH peptides (in the range of the receptors) and may play an important role in the modulation of CRH bioavailability (see Behan *et al.,* 1996). Analyses of the primary structures of the vertebrate CRH-BPs reveal a protein with high evolutionary conservation, which suggests strong selective pressure to maintain its structure and function (see Valverde and Denver, 1999, also unpublished data). In mammals, CRH-BP is expressed in multiple tissues, including the liver, brain, and pituitary gland. CRHBP circulates in the blood in humans but not in rats, which may be explained by the lack of expression in rat liver. The *X. laevis* CRH-BP was originally isolated from a subtractive-tadpole-tail cDNA library as a T_3-regulated gene (Brown *et al.,* 1996). We found that this gene is expressed in the frog brain, intestine, liver, and pituitary (Valverde and Denver, 1999) and preliminary data suggest that it is expressed in several other tissues (e.g.,

Fig. 6

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the gonads and skin; R. A. Valverde and R. J. Denver, unpublished). The role that this protein plays in modulating CRH action in any species is poorly understood, and few comparative studies in nonmammalian species have been done (see Valverde and Denver, 1999). The protein could modulate CRH action by binding it, and thus blocking its availability to receptors, or by targeting the peptide for clearance, as has been proposed in humans (Behan *et al.,* 1996). Alternatively, the CRH-BP might serve to maintain high concentrations of CRH in tissues or in tissue fluids, perhaps facilitating CRH action. Brown and colleagues (1996) suggested that the up-regulation of this protein during metamorphic climax might serve a negative feedback function by sequestering CRH and thus modulating its bioavailability; this hypothesis has not been tested.

4. Other Neurohormones Regulating Thyroid-Stimulating Hormone

Although CRH is the only hypophysiotropic peptide known to stimulate TSH release in tadpoles, the possibility that other hypothalamic hormones regulate TSH must be considered. Gonadotropin-releasing hormone (GnRH) was found to stimulate the thyroid axis in axolotls and adult frogs (Jacobs *et al.,* 1988b; Jacobs and Kuhn, 1988), acting directly on the pituitary gland (Denver, 1988). The physiological significance of this finding is unknown, and it is also unknown whether GnRH is stimulatory to TSH during the larval stage.

IV. ENDOCRINOLOGY OF PAEDOMORPHOSIS

A. Overview

Paedomorphosis is common among salamanders. Four out of nine families of salamanders are entirely paedomorphic, and the five other urodele families contain at least one paedomorphic species (Duellman and Trueb, 1994). Although there is a high frequency of paedomorphosis among salamanders, its physiological basis remains poorly understood (but see Rosenkilde and Ussing, 1996). To understand the underlying physiological mechanisms controlling paedomorphosis, two separate endocrine pathways must be considered. As described in Section III, metamorphosis in anurans is driven by the activation of the thyroid axis. This also appears to be the case in facultative paedomorphic salamanders. Because paedomorphs become sexually mature while remaining in the larval habitat, we discuss here the endocrine pathways controlling gonadal development and maturation and their possible interaction with hormones controlling metamorphosis.

B. Thyroid Axis

1. Facultative Paedomorphs

In facultative paedomorphic salamanders, metamorphosis can be induced by exposure to exogenous T_3 or T_4 (Norris and Platt, 1974). The injection of mammalian TSH can also induce metamorphosis in paedomorphs (Norris *et al.,* 1973). Because both the peripheral tissues and the thyroid gland are competent to respond to hormonal stimulation, it has been suggested that the failure to metamorphose results from the lack of stimulation of the secretion of pituitary hormones by hypothalamic neurohormones. Norris and Gern (1976) provided evidence that the lack of metamorphosis in facultative paedomorphs results from insufficient hypothalamic development. They induced metamorphosis in paedomorphic *A. tigrinum* salamanders by intrahypothalamic administration of T_4; intraperitoneal injection of the same dose did not induce metamorphosis. Intrahypothalamic administration led to an activation of the thyroid axis not seen after systemic administration, apparently as a result of the differentiation of the hypothalamic neurosecretory system (Norris and Gern, 1976).

TRH injections did not accelerate metamorphosis when administered intrahypothalamically (Norris, 1978). However, similar to anurans (discussed in Section III), later studies suggested that TRH is not active in TSH secretion in larval salamanders (see Darras and Kuhn, 1983; Jacobs and Kuhn, 1987). We found that CRH injections in larval *A. tigrinum* (derived from a facultative paedomorphic population in Michigan) accelerated metamorphosis (see Fig. 7). Experiments with CRH in paedomorphic animals are necessary to determine whether this neurohormone can induce such animals to metamorphose.

2. Obligate Paedomorphs

Obligate paedomorphs can be further divided based on a species' response to TH treatment. Although all

obligate paedomorphs do not metamorphose in the wild, inducible obligates do metamorphose when treated with TH. Permanent obligates, on the other hand, are insensitive to TH treatment (Wakahara, 1996).

The best-studied inducible obligate is the axolotl, *Ambystoma mexicanum*. It appears that low levels of circulating TH, low 5′-deiodinase activity, and low receptor number all contribute to the obligate paedomorphic lifestyle of the axolotl (Galton, 1992). The axolotl thyroid gland contains a large amount of thyroglobulin and is competent to respond to stimulation by TSH (Jacobs *et al.,* 1988a). The presence of an axolotl pituitary protein that has potent TSH-like activity has been demonstrated (Schultheiss, 1980). Thus, the paedomorphic state seems to be a result of the axolotl's lack of TSH release. Whether this is due to lack of hypothalamic stimulation, the presence of inhibitory factors, or an inability of the pituitary to respond to stimulatory neurohormones is uncertain.

Permanent obligates fail to respond to TH. This finding led to the hypothesis that TRs are not expressed or are somehow defective in these species. Partial TRα and TRβ sequences have been isolated from two members of the permanent obligate family Proteidae, Mudpuppy *(Necturus maculosus)* and *Proteus anguinus* (Safi *et al.,* 1997). Reverse transcriptase polymerase chain reaction (RT-PCR) analyses failed to demonstrate TRβ expression in *N. maculosus* (Safi *et al.,* 1997). The authors suggested that failure to express this receptor may explain the insensitivity to TH in *Necturus*. Although both TRα and TRβ genes were found in *Proteus,* expression studies were not conducted on this species. The TR sequences of both *Necturus* and *Proteus* were found to contain nonconservative mutations that could potentially affect the hormone-binding domain (although, the binding affinities of these receptors for THs have not been examined).

C. Reproductive Development

Although sex determination and primary sexual differentiation occur during embryonic and larval development in amphibians (see Hayes, 1998, for review), many species do not reach sexual maturity until several years after metamorphosis (Duellman and Trueb, 1994). For most amphibians, the transition from the larval to the postmetamorphic form must occur before the initiation of maturation. Sexual maturation is arrested until a certain age when proper body size, body weight, or stage of nervous system development allows for the final stages of sexual maturation. Ryan and Semlitsch (1998) proposed that the decoupling of metamorphosis and sexual maturation has allowed paedomorphosis to evolve. The physiological mechanisms and environmental influences that contribute to the initiation of maturation in paedomorphic salamanders are probably very similar to those in amphibians that mature after metamorphosis.

The onset of sexual maturation is controlled by poorly understood central nervous system mechanisms that lead to the activation of GnRH neurons in the hypothalamus. GnRH acts on the anterior pituitary, which secretes (GtHs), LH, and FSH. The GtHs act on the gonads to bring about gonadal maturation and sex steroid secretion. In males, GtHs increase testosterone production, promote spermatogenesis, and increase testicular size by promoting the growth of interstitial cells and seminiferous tubules. In females, GtHs are responsible for promoting ovarian growth, sex steroid production, and oocyte maturation (Jorgensen, 1992). Circulating GtHs are also required for the ongoing maintenance of gametogenesis and reproductive structures. Hypophysectomy of sexually mature amphibians results in the degradation of vitellogenic oocytes (Lofts, 1974), arrested spermatogenesis, and degeneration of seminiferous tubules (Guha and Jorgensen, 1978a). Replacement of GtHs in hypophysectimized animals restores gonadal function in both males and females (Jorgensen, 1975; Guha and Jorgensen, 1978b).

Although the central mechanisms controlling the onset of maturation in amphibians are incompletely understood, nutritional status and growth rates are important factors. These relationships have been studied both in nature and in the laboratory. The high-altitude plethodontid salamander, *Bolitoglossa subpalmata,* grows in its native habitat at a rate of $<$0.5 mm/month. In the laboratory at their preferred temperature (10°C) and fed *ad libitum,* salamanders grew 1.3 mm/month. The growth rates of wild and lab-reared animals were positively correlated with the timing of sexual maturation. Laboratory-reared males matured at 1.5 years of age and females at 3 years of age, compared to 6 and 12 years for wild males and females, respectively (Houck, 1982).

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Sexual maturation is typically correlated with an abrupt decline in somatic growth in anurans (Hemelaar, 1988) and in urodeles (Tiley, 1980). This is especially apparent in females, in which somatic growth may approach zero concurrent with the initiation of the first bout of reproduction (Jorgensen, 1986a). Vitellogenesis represents a large maternal investment; in gravid females, the ovaries may constitute 20–30% of body mass. Thus, during vitellogenesis energy is reallocated from somatic growth to reproduction (Jorgensen, 1986a). In males, sexual maturation and somatic growth are less tightly coupled because of the significantly lower investment that males must make in testicular development (Jorgensen, 1986a). Mature gonads in males contribute less than 1% to total body mass.

There may be a functional linkage between fat stores (which are dependant on nutritional status) and gonadal development in amphibia. Fat bodies are located in close proximity to the gonads in amphibians and an inverse relationship has been demonstrated between the size of the fat body and the size and stage of development of testes or ovaries in both anurans and urodeles (Fitzpatrick, 1976). Fat bodies are greatly reduced in size in sexually mature amphibians, whereas the fat bodies of immature amphibians are comparatively larger in size (Fitzpatrick, 1976).

Investigations into a possible linkage between fat bodies and gonads involved excising fat bodies and examining the effects on the gonads. In the newt *Notophthalmus viridescens,* fat-body removal caused the degeneration of gametes in both males and females (Adams and Rae, 1929). In the salamander *Amphiuma means,* fat-body removal prevented vitellogenic growth of the oocytes (Rose, 1968). Removal of fat bodies in anurans had a similar effect on gonads in males (Kobayashi and Iwasawa, 1984) and females (Pierantoni *et al.,* 1983). Unilateral excision of the fat body affected gametogenesis only in the gonad located on same side from which the fat body was removed; gametogenesis proceeded normally in the contralateral gonad, where the fat body remained intact (Adams and Rae, 1929). These findings prompted the hypothesis that a local mechanism mediated the fat–gonadal interaction. However, careful investigation of the vascularization revealed that there are independent blood supplies for the fat bodies and gonads and that fat bodies of some species spread along blood vessels near the ovary (Jorgensen, 1986b). Thus, there is the possibility that impaired gonadal development in these experiments was due to an experimental artifact, related to the disruption of blood supply to the gonad, rather than the removal of signals originating in the fat body.

Although the relationship between the amphibian fat bodies and the gonads is unclear, the fact remains that fat bodies tend to be large in immature animals and smaller in mature animals. This correlation could reflect the importance of fat stores for sexual maturation. Similarly, plenthodontid salamanders, who store fat in their tails, show a correlation between tail size and oocyte size, possibly reflecting the size of nutrient stores (Fraser, 1980). Removal of the tail prevented sexual maturation in these animals (Maiorana, 1977).

Although nutritional status is important for determining the onset of maturation, the physiological signal that communicates that there is ample energy for sexual maturation is unknown. A polypeptide called leptin, secreted by fat tissue in mammals, has received considerable interest (for review, see Houseknecht *et al.,* 1998; Wauters *et al.,* 2000). Leptin injections into female mice reduced food intake by 20 percent, but accelerated all measured indices of maturation (e.g., age at first estrus, ovarian weight, and ovulatory index) compared to pair-fed control animals. Animals fed *ad libitum* exhibited the same timing of events as leptin-injected animals (Cheung *et al.,* 1997). These findings and others have led to the hypothesis that leptin, which is released in greater amounts as fat stores increase (Houseknecht *et al.,* 1998; Wauters *et al.,* 2000), signals positive energy balance and thus brings about the onset of sexual maturation (i.e., puberty). However, it is unclear whether leptin serves as a trigger for the onset of sexual maturation or acts in a permissive manner. The presence or absence of such a hormone in amphibians has not, to our knowledge, been studied. Studies in lizards *(Sceloporus undulatus)* show that injections of mammalian leptin can increase metabolic rates, lower food intake, and increase body temperature (Niewiarowski *et al.,* 2000).

Hormones involved in regulating growth may also be important for the onset of maturation. As discussed in Section III, GH is important for growth of adult structures, but probably does not play a significant role in larval amphibians. Based on expression analyses

and functional studies (see Section III), it is unclear whether PRL plays a role in controlling growth in larval amphibians. To our knowledge, PRL expression in paedomorphs has not been analyzed. It is uncertain which hormones play important roles in controlling larval growth in amphibians.

V. INTEGRATING EVOLUTION, ECOLOGY, AND ENDOCRINOLOGY

A. Metamorphosis

The activity of the thyroid axis in tadpoles can be regulated at multiple levels (see Section III; Fig. 5) and this activity ultimately 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 may play a critical role in transducing environmental information and regulating metamorphic timing. From a developmental and physiological perspective, the upper and lower limits to the larval period in different species is established genetically through programming the developmental schedules for each of the components of the endocrine system (the establishment of functional endocrine cells and tissue competence to respond to thyroid and steroid hormones) and epigenetically through the regulated secretion, metabolism, and action of hormones. The environment could impact the developmental schedules and most certainly impacts the production and perhaps the actions of the hormones. Also, antagonism between growth-promoting hormones and morphogenic hormones might underlie the trade-offs between growth rate and development rate.

Fig. 5

Few studies have addressed these issues from an integrative perspective (physiology and ecology). Here we discuss these issues and develop several hypotheses to explain how the limits to the larval period are established (in a physiological and developmental sense) and how plasticity in metamorphic timing within those limits is controlled.

1. Limits to the Length of the Larval Period

Why do amphibians differ in the lower and upper limits to the lengths of their larval periods and what determines tadpole growth and development rates and size at transformation? How does the timing of metamorphosis evolve? Few studies have attempted to address natural selection for the timing of metamorphosis; however, there is strong correlative evidence for the hypothesis that the length of the larval period is a reflection of the characteristics of the ancestral habitat (permanence and predictability, resource availability and competition, thermal environment, predation, etc.). The most important variable in this equation is likely to be habitat permanence because amphibian larvae depend on an aquatic environment for growth and development. It is also important to consider how factors operating in both life history stages (larval and adult) influence selection for the timing of metamorphosis (see Werner, 1986). Such questions have been addressed by ecologists (see Section II). Here we consider the question: What specific physiological regulatory systems in amphibian larvae might be targets for selection?

a) Lower Limit The earliest time at which tadpoles initiate metamorphosis in nature is probably influenced by the animal's size and the environmental conditions. But what determines the earliest possible time that a tadpole can enter metamorphosis and why does this timing differ among species? As discussed in Section II, the Wilbur-Collins model (Wilbur and Collins, 1973) proposes that tadpoles must reach a minimum body size before metamorphosis is possible. Thus, there must be a lower size limit, below which metamorphosis is impossible. This lower limit results from morphological and physiological constraints; for example, prey-catching ability and the size of prey, susceptibility to desiccation (higher surface-to-volume ratio of smaller animals) and susceptibility to predation, among others. Clearly, amphibians show considerable variation in the lower size limits for metamorphosis. For example, some species such as *Pseudacris* and *Bufo* grow very little during the larval phase and thus metamorphose at a very small size (7–9 mm snout to vent length), whereas others exhibit considerable growth and metamorphose at a large size (e.g., *Rana catesbeiana,* 20–60 mm snout to vent length; reviewed by Werner, 1986).

Is the minimum taxon-specific size for metamorphosis correlated with the establishment of competence to respond to metamorphic hormones? In *X. laevis* the capacity to respond to TH (i.e., increased RNA and protein

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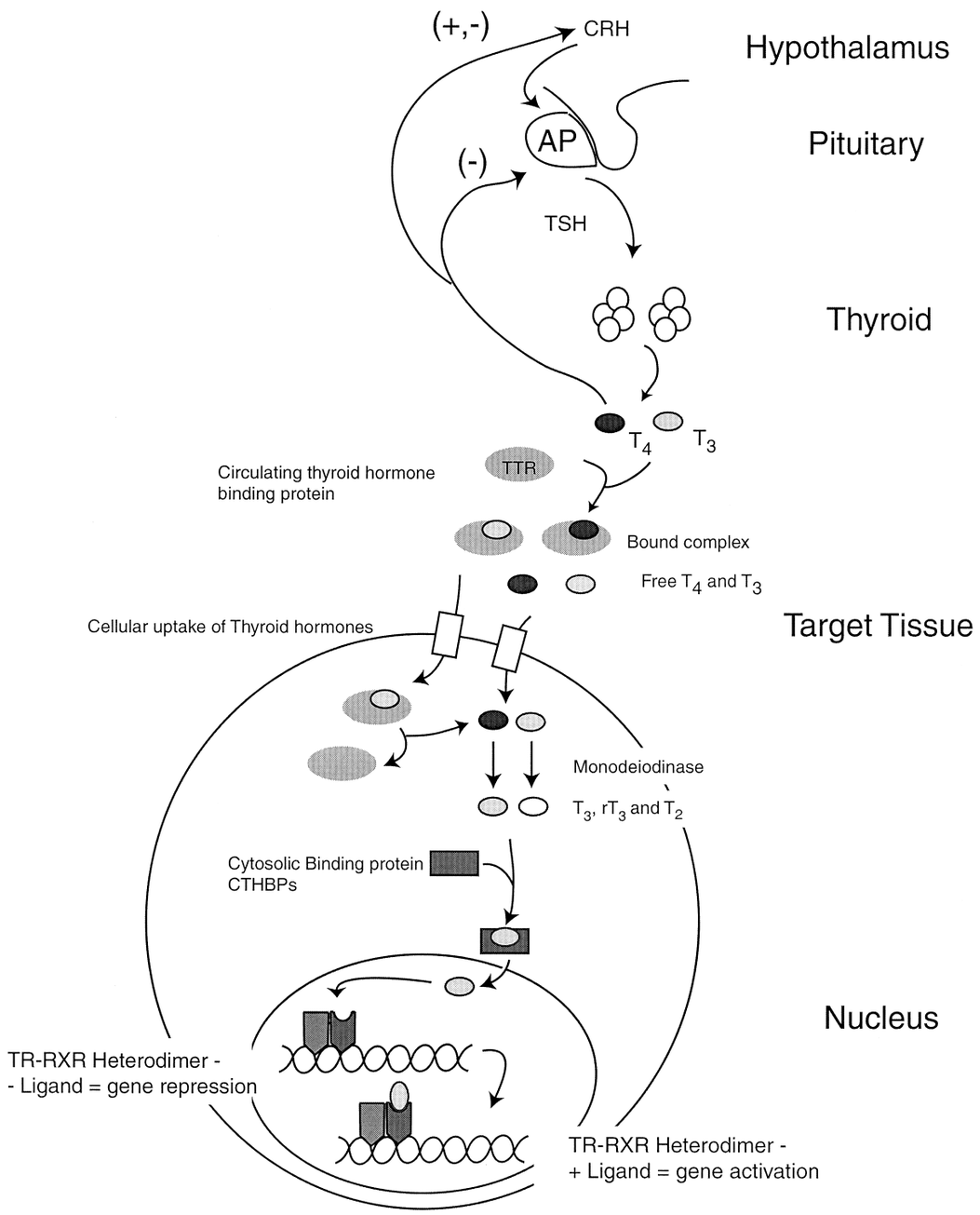

FIGURE 5 The integrated thyroid axis. Environmental regulation of the thyroid axis occurs at multiple levels. AP, anterior pituitary; CRH, corticotropin-releasing factor; RXR, retinoid-X receptor; T_3, 3,5,3′-triiodothyronine; T_4, thyroxine; TR, thyroid hormone receptor; TSH, thyroid-stimulating hormone; TTR, transthyretin. Minuses indicate a negative feedback. In the case of T_4 and T_3 effects on the brain, (+/−) indicates that these hormones promote differentiation of neurosecretory centers (and other brain regions) in addition to their negative feedback effects on neurohormone and pituitary hormone secretion.

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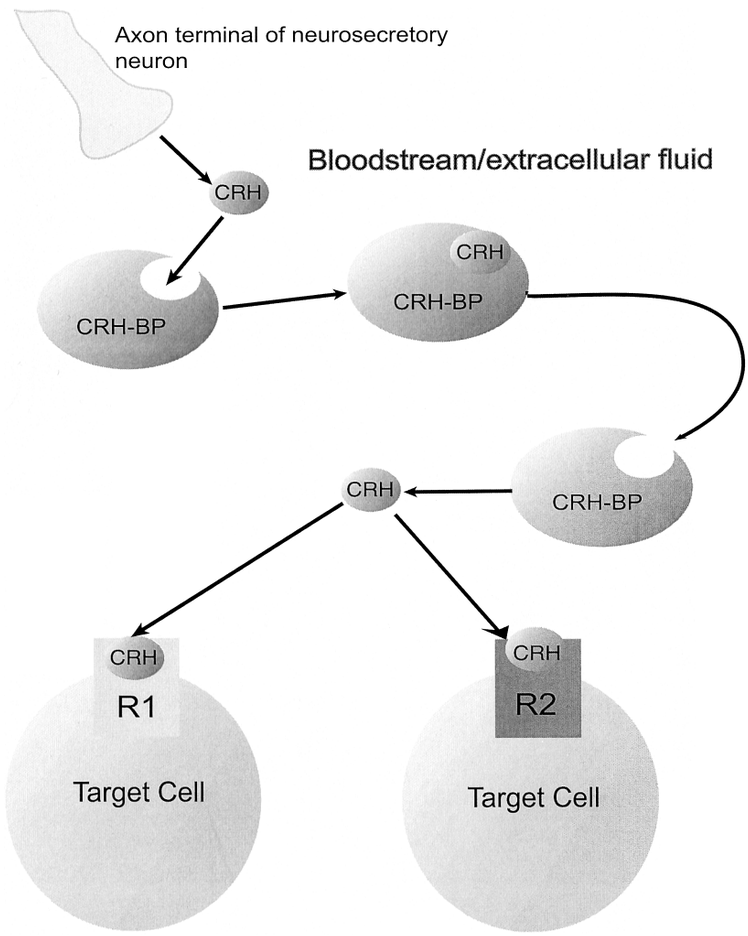

FIGURE 6 Regulation of CRH bioavailability by CRH binding protein (CRH-BP). Schematic representation of CRH interactions with its binding protein and two receptor subtypes.

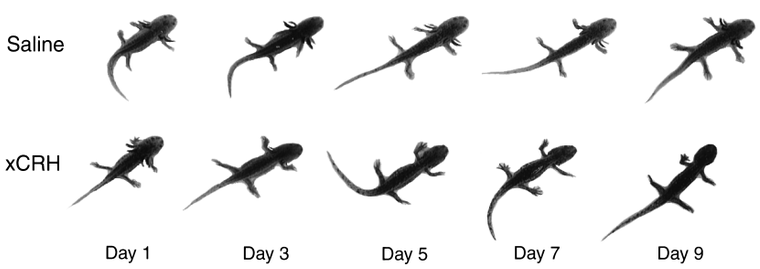

FIGURE 7 Effects CRH on metamorphosis in *Ambystoma tigrinum*. Injections of ovine CRH (oCRH, 1 μg, i.p.) or saline (0.6%) were administered daily ($n = 6$). Digital images were captured every other day to access metamorphic progression. Note the gill resorption in larvae receiving oCRH injections.

synthesis) is established early, just after hatching (see Tata, 1968). Thus, competence to respond to TH is established well before the minimum size for normal metamorphosis is reached. Is the minimum size correlated with the establishment of competence to produce metamorphic hormones in sufficient quantities to drive morphogenesis? The capacity to up-regulate hormone production takes longer to develop and depends on the maturation of the neuroendocrine system (reviewed by Denver, 1996).

There is considerable variation among species in the time it takes to proceed from hatching to the first appearance of limb buds (premetamorphosis), and then from limb bud appearance to late prometamorphosis. These two periods are likely to be independent targets for selection. During the premetamorphic period, selection for growth rate may be most important. Plasticity in the length of the premetamorphic period depends primarily on growth opportunities, and tadpoles have no choice but to make a living in the larval habitat and attain the minimum size for metamorphosis. During the prometamorphic period, selection for development of the endocrine system is probably the more important factor. During this period, a tadpole's endocrine system is sufficiently developed to allow it to make developmental decisions. That is, if conditions are favorable, the rate of TH production remains low and tadpoles continues to capitalize on favorable growth conditions. If conditions deteriorate, tadpoles have the capacity to activate endocrine systems and transition from the aquatic to the terrestrial habitat.

Where is the metamorphic clock located? Etkin (1970) argued that the clock is located in the hypothalamus. For example, autotransplantation of the pituitary primordium to the tail of the frog embryo (separation from stimulatory control by the hypothalamus) results in more rapid growth compared with controls and a failure to metamorphose (see Etkin, 1970). Destruction of the preoptic nucleus or surgical removal of the primordium of the posterior hypothalamus (and thus isolation of the pituitary from the brain) prevents metamorphosis (reviewed by Denver, 1996). Studies of the normal development of the neurosecretory centers of the hypothalamus and the median eminence further support this hypothesis (see Etkin, 1970).

Although the neuroendocrine system is likely to be central to the control of metamorphic timing, this timing may be influenced at other levels. Other sites of regulation might involve the production of hormone transport proteins such as TTR, tissue monodeiodinases, membrane TH transporters, and CTH-BPs (see Fig. 5; discussion in Section III). We know so little about the roles of these proteins (with the exception of the monodeiodinases) in metamorphosis and the regulation of their biosynthesis that it is difficult to predict whether their regulation is an important site for the control of metamorphic timing.

A potentially important site for the regulation of metamorphic timing is at the level of hormone receptor synthesis. The timing of the expression of receptors for neurohormones in the pituitary gland may be regulated, but this has not been studied in any amphibian. We know that TH receptor expression, primarily TRβ, is up-regulated during prometamorphosis and that this up-regulation depends on TH. The hormone must be present for the receptors to be expressed and to function, and the finding of TR autoinduction supports the view that the clock is in the hypothalamus where hormone production is controlled. Other possible points of regulation of TR action include the expression of RXRs, and coactivator and corepressor proteins.

In conclusion, although several sites for regulation are possible, evidence suggests that the primary clock that determines when a tadpole can enter metamorphosis is related to the degree of development of the neuroendocrine system.

b) Upper Limit An environment with good growth conditions and low predation favors a longer larval period in most species; under such circumstances tadpoles would be expected to push the upper limit. But even if we maintain tadpoles in the laboratory under constant favorable conditions they do ultimately metamorphose; they won't grow indefinitely. What physiological or developmental mechanism is responsible for the spontaneous activation of the endocrine system controlling metamorphosis? Perhaps the slow increase in thyroid activity eventually reaches a threshold such that the system is pushed into metamorphic climax. The better the conditions, the lower the thyroid activity, but it eventually reaches a level where positive feedback is initiated. Or perhaps the activation follows from the animal reaching some upper size limit, and the

subsequent decline in growth-promoting hormones removes antagonism on the thyroid system. Because anurans are not paedomorphic, the costs of remaining in the larval habitat longer should eventually outweigh the benefits of larger size at metamorphosis.

2. Plasticity in the Timing of Metamorphosis

Within the lower and upper limits to the larval period, tadpoles exhibit considerable plasticity in their timing of metamorphosis. This phenotypic plasticity depends on environmental factors, that is, the quality and suitability of the larval habitat for growth and survival. The majority of amphibians that have been studied exhibit phenotypic plasticity within the limits of the length of the larval period, rather than exhibiting a fixed rate of development. Here we address the question: What physiological systems enable plasticity in the timing of development and are thus targets for selection?

a) Integrated Endocrine System Controlling Metamorphosis and Potential Loci for Environmental Modification of Endocrine Activity Points of regulation by the environment might include the neuroendocrine system, peripheral endocrine organs, hormone transport and metabolism, and hormone action. But how are environmental factors sensed? Thermal, osmotic, and effects related to the gaseous environment could be sensed directly by most or all tissues. The influence of other factors, such as photoperiod, resource availability, predator presence, and crowding are likely integrated by the neuroendocrine system and transduced by the hypothalamus into changes in peripheral endocrine gland activity.

The availability of biologically active hormone is regulated in tissues by the monodeiodinases and the expression of these enzymes could be modified either directly or indirectly by environmental factors. An example of indirect regulation of monodeiodinases by environmental factors is by corticoids, which have been shown to increase 5′-deiondinase activity with the result that more of the active hormone T_3 is generated. This regulatory relationship might indicate that stress and stress hormones can accelerate metamorphosis by upregulating 5′-deiondinase (see Section III). Similarly, TR synthesis might be regulated directly or indirectly by environmental factors, which would then influence metamorphic timing. There is little known about which factors, physiological or environmental, regulate nuclear receptor expression in any species. As for monodeiodinase, evidence suggests that the corticoids can enhance TH action by up-regulating TR expression, and so TR biosynthesis is an additional site where stress and stress hormones may modulate timing (see Section III).

b) Plasticity Mediated by the Neuroendocrine System As described, the neuroendocrine system is likely to be the clock regulating spontaneous metamorphic timing. Furthermore, the external and internal environments can modify the activity of the neuroendocrine system. Many biotic and abiotic environmental factors are detected by animal sensory systems, integrated in higher brain centers, and then transduced via the neuroendocrine system.

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 (see Section II; Table 1). This is especially true for desert amphibians that tend to breed in ephemeral habitats. We have studied the phenotypic and physiological responses of tadpoles of the western spadefoot toad *(S. hammondii)* to pond drying in the laboratory (see Section II).

As described in Section III, injections of CRH-like peptides accelerated metamorphosis in tadpoles of several amphibian species including the western spadefoot toad. Conversely, we found that the developmental acceleration induced by water-volume reduction could be attenuated by the treatment of tadpoles with the CRH receptor antagonist α-helical $CRH_{(9-41)}$ or by passive immunization with anti-CRH serum. Furthermore, spadefoot toad tadpoles had elevated hypothalamic CRH content at the time that they first responded (morphologically and endocrinologically) to the water-volume reduction in the laboratory (Denver, 1997a); these tadpoles also exhibited a precocious elevation in whole-body TH and corticosterone contents (Denver, 1998). Because the secretion of CRH is activated by stress, we hypothesized that CRH may play a central role in mediating a tadpole's developmental response to a deteriorating larval habitat. We also proposed that CRH may represent a phylogenetically ancient developmental cue that vertebrates use to assess changes

in their habitat and to mount an appropriate developmental and physiological response. We based this hypothesis on data from mammals that show that CRH of fetal or placental origin controls the timing of the length of gestation and may shorten the gestational period under conditions of fetal stress (Smith, 1998).

But do other environmental factors that are known to alter the timing of metamorphosis also act through the neuroendocrine stress axis? We have observed elevated whole-body corticosterone content in *R. pipiens* tadpoles fed limited resources or subjected to high conspecific density, compared to their high-resource low-density counterparts (Glennemeier and Denver, in press). 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; see Section II). This slowed growth caused by crowding stress was reversed in tadpoles by treatment with the corticosterone-synthesis inhibitor metyrapone, again suggesting a functional role for the hypothalamic-hypophyseal-interrenal axis in mediating the larval developmental response to environmental conditions (Glennemeier and Denver, in press). Hayes (1997) also reported an elevation in whole-body corticosterone content in *B. boreas* tadpoles caused by crowding. 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.

B. Facultative Paedomorphosis

How (in an ultimate sense) are different life history trajectories selected (paedomorphosis vs metamorphosis)? Why (in a proximate sense) do some animals become paedomorphic, whereas others become metamorphic? The life history trajectory is influenced by the environment, but ultimately expressed via a change in hormone production and action. The components of the endocrine system are likely to be targets for selection.

As already discussed, the neuroendocrine system serves to transduce many environmental factors into changes in development and physiology. To understand the physiological processes involved in the determination, development, and maintenance of each morphology, two separate antagonistic developmental pathways must be considered. Metamorphs activate their thyroid axis, which results in the morphological changes necessary for adaptation to the terrestrial habitat. Paedomorphs have increased activity of the hypothalamic-pituitary-gonadal axis, which results in precocious sexual maturation. In the discussion that follows we address the possibility that interactions between the thyroid and gonadal axes may underlie the mechanism of the selection of the life history trajectory of a facultative paedomorphic salamander.

1. Integrated Organismal Responses to the Environment—Metamorphosis vs Paedomorphosis

Metamorphosis allows developing larvae to escape a deteriorating aquatic habitat and move into the terrestrial habitat. The environmental conditions that trigger metamorphosis (discussed in Section II) in facultative paedomorphic salamanders probably do so by activating neuroendocrine pathways, as discussed previously for anurans. In support of this hypothesis, we observed that injections of the stress neuropeptide CRH accelerated metamorphosis in larvae of the tiger salamander (*A. tigrinum;* Fig. 7; G. C. Boorse and R. J. Denver, **Fig. 7**
submitted). We also observed that captured tiger salamander larvae metamorphose within 2 days of transfer to the laboratory (R. J. Denver, unpublished observations). Earl Werner (personal communication) found that several paedomorphic *A. tigrinum* brought into the laboratory from experimental ponds (at the E. S. George Reserve, Pinckney, MI) quickly metamorphosed. That they were indeed paedomorphs was verified by dissection, which showed scars on the ovaries, indicating that reproduction in the larval stage had occurred. We interpret these anecdotal observations as evidence for capture-stress-induced activation of the endocrine system controlling metamorphosis.

Limitations in food availability result in trade-offs among allocation of energy to maintenance, growth, reproduction, and storage. We hypothesize that a favorable nutritional state of facultatively paedomorphic salamanders allows for early age at maturity. Adequate nutritional status appears to be a major factor in determining the onset of sexual maturation (see Section IV). The environmental conditions that produce paedomorphs presumably have higher per capita resources and less competition among larvae (Scott, 1993; Ryan and Semlitsch, 1998)—low larval density corresponds to lower interference rates or less crowding of larvae; constant water level provides stable larval density and stable resources. Considerable time and energy must be invested in the extensive morphological and biochemical restructuring that takes place during metamorphosis. Because paedomorphs do not undergo metamorphosis, they do not have this additional energetic cost.

The effects of differential resource allocation during larval development have not been studied in paedomorphic salamanders, but studies in metamorphic species have demonstrated that larvae allocate resources differently depending on environmental conditions, which then affects both growth and reproduction. Marbled salamander *(Ambystoma opacum)* larvae maintained at low densities had higher lipid stores at metamorphosis and exhibited longer survival in captivity. Lower larval densities also translated into benefits for the terrestrial adult as demonstrated by their larger size at first reproduction, earlier age at first reproduction, and greater clutch size (Scott, 1994). Juvenile *A. opacum* females exposed to high food levels exhibited larger size, higher lipid levels, larger clutch size, and earlier age at first reproduction than juveniles exposed to medium or low food levels (Scott and Fore, 1995). Each of these traits can contribute to an individual's overall lifetime reproductive success.

2. *Physiological and Ecological Trade-offs between Metamorphosis and Paedomorphosis*

The environment determines the life history trajectory by influencing the activity of endocrine glands. We hypothesize that there are antagonistic interactions among the endocrine pathways controlling growth, metamorphosis, and sexual maturation, and the strength of these interactions underlies the choice of developmental pathway taken.

a) Thyroid Axis Inhibition of Gonadal Development

The cost of morphological and biochemical restructuring during metamorphosis decreases the amount of resources available for gonadal development in metamorphs. The thyroid and interrenal hormones that drive metamorphosis may also directly inhibit gonadal development. Both stress hormones and THs have been demonstrated to inhibit gonadal function in vertebrates.

Stress is well known to inhibit reproduction in all vertebrate taxa. Stress hormones can influence sexual function at all levels of the hypothalamic-pituitary-gonadal axis: the hypothalamus to inhibit GnRH release, the pituitary to interfere with GnRH-induced LH and FSH release, and the gonads to alter the stimulatory effect of GtHs on sex steroid secretion (Rivier and Rivest, 1991) (Fig. 8). Both CRH and glucocorticoids are known to inhibit gonadal activity. Intracerebroventricular injections of CRH inhibited GnRH secretion in rodents and primates, whereas administration of CRHR antagonists reversed the inhibitory effects of stress on GnRH (Rivier *et al.,* 1986). Elevated plasma glucocorticoid concentrations also reduced GnRH secretion (Suter and Schwartz, 1985). Corticoid receptors and, surprisingly, CRH mRNA are expressed in rodent gonads, and CRH has been shown to directly inhibit steroidogenesis (Saez *et al.,* 1977; Ulisse *et al.,* 1989; Fabbri *et al.,* 1990). The stress hormones may directly inhibit sex steroidogenesis by reducing sensitivity to GtH stimulation (Charpenet *et al.,* 1981, 1982). **Fig. 8**

The activation of the stress hormonal axis in metamorphic salamanders may result in the inhibition of sexual maturation. Examples of such actions in amphibia include studies in adult *X. laevis* in which crowding and underfeeding reduced ovarian growth (Alexander and Bellerby, 1938); such effects could be reversed by injections of human chorionic gonadotropin (hCG), even in toads that were emaciated from starvation (Jorgensen, 1982). The stress to the frogs of being captured and brought into the lab caused a threefold increase in atresia of vitellogenic oocytes, which was attributed to a decrease in circulating GtHs (Pancharatna and Saidapur, 1992). These findings lead to the hypothesis that elevated stress hormones in metamorphs delays gonadal development.

Increasing plasma TH concentrations during metamorphosis may also play an important role in inhibiting

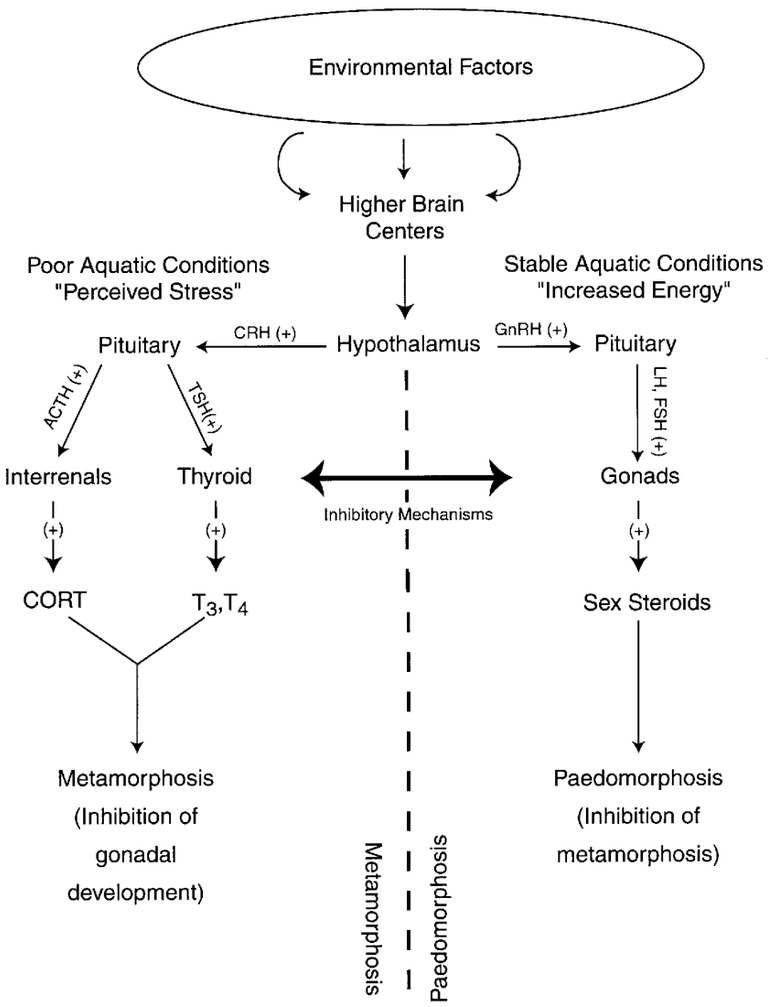

FIGURE 8 Environmental regulation of hormonal axes controlling metamorphosis and paedomorphosis in facultatively paedomorphic salamanders. Pluses indicate a stimulatory effect. Components of a given pathway (metamorphosis vs paedomorphosis) may inhibit the development of the alternative morphology. ACTH, adrenocorticotropin; CORT, corticosterone; CRH, corticotropin-releasing factor; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; T_3, 3,5,3′-triiodothyronine; T_4, thyroxine; TSH, thyroid-stimulating hormone.

gonadal development. Wakahara (1994) treated larvae of the facultative paedomorphic salamander *Hynobius retardatus* with a goitrogen (antithyroid compound) and this both inhibited metamorphosis (as expected) and caused accelerated spermatogenesis and increased testicular growth (Wakahara, 1994). These findings suggest that TH exerts an inhibitory action on gonadal development in these animals. The mechanism by which TH inhibits gonadal development is unknown. However, it may be conserved across vertebrate taxa because goitrogen treatment of neonatal rats resulted in an increase in adult testis size (Cooke and Meisami, 1991).

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Thus, both TH and stress hormones have been shown to inhibit gonadal development. Elevation in the production of these hormones at metamorphosis may explain why gonadal development is delayed in metamorphs compared with paedomorphs.

b) Sex Steroid Inhibition of the Thyroid Axis Facultative paedomorphs retain the ability to metamorphose, which is important for escaping mortality in a deteriorating aquatic habitat. However, if an individual matures early and then undergoes metamorphosis, the benefits of increased survival and higher quality offspring are lost because the age at first reproduction is the same as other metamorphs. Early maturation may then carry significant costs such as reduced body size or reduced parental investment at time of reproduction (Stearns, 1991).

We hypothesize that because paedomorphs carry an added cost associated with metamorphosis, especially if metamorphosis occurs before first reproduction, paedomorphs may not metamorphose as readily as sexually immature larvae. Paedomorphs are expected to delay metamorphosis and remain in a deteriorating habitat until the cost of remaining in the aquatic habitat is greater than the cost of metamorphosis. Paedomorphs may be less sensitive to metamorphic hormones; for example, sexually mature larvae of *A. tigrinum* required higher doses of exogenous TH to induce metamorphosis than immature larvae (Norris and Platt, 1974). We predict that elevated plasma concentrations of sex steroids in the paedomorph reduce its likelihood of metamorphosing by decreasing sensitivity to metamorphic hormones. Although sex steroids have not been quantified in paedomorphs, sex steroid production is presumably higher than in sexually immature larvae or metamorphic juveniles. Paedomorphs exhibit well-developed mature gonads in both males and females that require circulating gonadotropins for their maintenance (Jorgensen, 1975; Guha and Jorgensen, 1978b). Male paedomorphs exhibit secondary sexual characteristics (i.e., enlarged cloaca) that are dependent on elevated plasma testosterone concentrations (Norris *et al.,* 1989).

Both testosterone and estradiol inhibit TH-induced metamorphosis in *X. laevis* (Gray and Janssens, 1990). The inhibitory effects were only seen *in vivo* and not *in vitro*. This suggests the inhibitory action of sex steroids is not at the peripheral tissues but at a higher level in the thyroid axis (Fig. 8). Sex steroids also inhibit metamorphosis in Japanese flounder (*Paralichthys olivaceus;* Dejesus *et al.,* 1992); metamorphosis in flounder is endocrinologically similar to amphibian metamorphosis (Dejesus *et al.,* 1993).

The initiation of sexual maturation in larvae is tightly coupled to nutritional status, a good indicator of environmental quality. Paedomorphosis in stable aquatic conditions allows for the continued exploitation of resources. Facultative paedomorphs (or immature larvae) may respond to a deteriorating habitat by activating neuroendocrine stress pathways (see previous discussion). Although sex steroids may suppress the metamorphic pathways, we predict that the effects of thyroid and interrenal hormones dominate the sex steroid effects. The neuroendocrine stress pathway may be an evolutionary-conserved mechanism that allows animals to monitor their environment and to respond to deleterious changes in habitat quality by metamorphosing.

The decision to metamorphose or to become paedomorphic and remain in the larval habitat can have critical fitness-related consequences. Understanding the relationship between fitness and the phenotypes produced by different environments will be necessary to evaluate such consequences. Approaches that attempt to place the mechanistic bases of development in an ecologically relevant context are essential for understanding the potential physiological constraints on the evolution of paedomorphosis and metamorphosis.

Acknowledgments

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