CHAPTER EIGHT

Salamander Paedomorphosis: Linking Thyroid Hormone to Life History and Life Cycle Evolution

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Abstract

Many salamanders have biphasic life cycles with aquatic larval and terrestrial adult phases. In these species, the transition between phases—metamorphosis—requires thyroid hormone (TH) activation of transcriptional programs that cause regression of larval traits and development of adult traits. During salamander evolution, TH signaling pathways have been altered in biphasic species to yield paedomorphic salamanders that retain larval traits and attain sexual maturity in larval aquatic habitats. We review literature concerning the ecology, evolution, and hormonal regulation of metamorphic, paedomorphic, and facultative salamander life histories. We then discuss recent microarray results that detail gene expression signatures of metamorphosis and paedomorphosis, and genetic results that establish TH responsiveness as a continuous trait with a quantitative trait locus (QTL) basis. TH-responsive QTL from ambystomatid salamanders explain variation in metamorphic timing, expression of metamorphosis versus paedomorphosis, and adult fitness traits. We propose a model for salamander life history evolution that links adaptation to aquatic habitats with TH-responsive loci that pleiotropically alter metamorphic timing and adult body size. Future studies that adopt

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genetic and genomic approaches will further establish salamanders as ideal models for investigating TH signaling mechanisms that regulate postembryonic development and the expression of alternate life histories.

Salamanders are one of three primary groups of amphibians, the other two being caecilians and anurans. Ancestrally, all three groups trace their origins to ancestors that present biphasic life cycles with an aquatic larval phase and a more terrestrial adult phase (Duellman & Trueb, 1986). However, alternate modes of development subsequently evolved within all three groups. Interestingly, ancestral vestiges of metamorphosis are observed during early development of direct developing anurans and salamanders (Callery & Elinson, 2000; Kerney, Blackburn, Muller, & Hanken, 2012), suggesting shared evolutionary potential for radical and early shifts in the timing of metamorphosis. Radical, later shifts in metamorphic timing are only observed in salamanders. In the most extreme cases, metamorphosis has been abolished completely, yielding bizarre larval-form adults with completely aquatic life cycles (Gould, 1977; Shaffer & Voss, 1996). These unique paedomorphic forms are ideal for investigating mechanisms of thyroid hormone (TH) regulation that are associated with adaptive delays in metamorphic timing and the evolution of novel life histories.

1. WHAT IS PAEDOMORPHOSIS AND WHY SALAMANDERS?

Paedomorphosis is a heterochronic term that describes a specific pattern of evolution, the retention of ancestral juvenile traits in the adult stage of a derived species (Gould, 1977). In this meaning, paedomorphosis references an evolutionary change in developmental timing between an ancestor and descendant species. However, paedomorphosis is often used in a more general sense to describe the retention of larval morphological traits in adult salamanders, irrespective of phylogeny. The evolution or expression of paedomorphosis is clearly associated with TH, the primary metamorphic hormone in anurans, salamanders, and some fish. In species that undergo a metamorphosis, TH induces the regression of larval traits and the development of traits typical of a more terrestrial adult (Fig. 8.1). As we discuss in more detail below, many paedomorphic salamanders can be induced to undergo partial or complete metamorphosis by simply placing them in a bath of TH. This suggests the possibility that paedomorphosis evolves "simply" by blocking the synthesis, secretion, or reception of TH in target cells (Page,

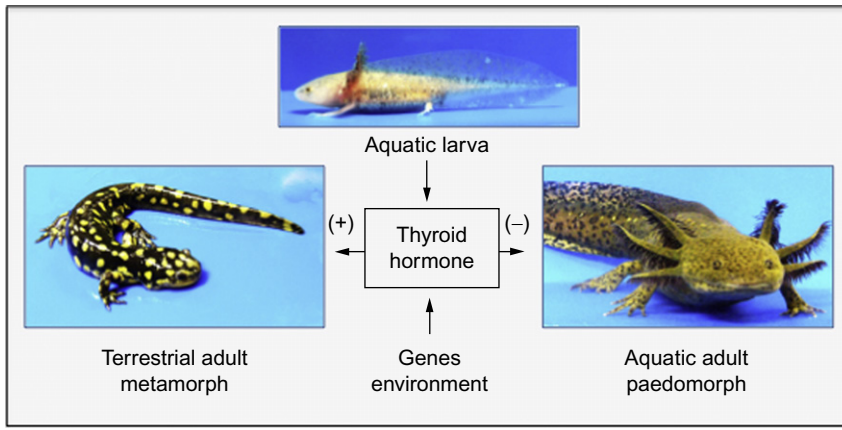

Figure 8.1 A representative salamander larva, adult metamorph (*Ambystoma tigrinum*), and adult paedomorph (*A. mexicanum*). Critical levels of thyroid hormone induce metamorphosis in salamanders. Environmental and genetic factors may cause thyroid hormone levels to be low, resulting in paedomorphic salamanders. *The pictures were taken by Jeramiah Smith.*

Boley, Smith, Putta, & Voss, 2010). However, TH secretion is regulated by complex, neuroenodrine pathways that must first develop during the larval period before these systems become competent to signal TH release in response to intrinsic and extrinsic cues (Denver, Glennemeier, & Boorse, 2002). Thus, paedomorphosis could conceivably evolve by altering the development or function of a number of different cells, tissues, and organs that regulate the release or reception of TH. Given this complexity and the broad pleiotropic role that TH assumes in amphibian development and physiology, it seems likely that paedomorph evolution requires multiple changes across many genes.

The specific heterochronic process most commonly associated with salamander paedomorphosis is neoteny (Gould, 1977)—relative to the ancestor or metamorphic condition, somatic development is delayed but rate or timing of reproductive maturation is the same. However, paedomorphosis in some species is associated with an earlier time to first reproduction, achieved by accelerating the rate of gonadal development (progenesis in *Triturus alpestris*; Denoel & Joly, 2000) or by initiating reproductive maturation earlier in the life cycle (peramorphosis in *Ambystoma talpoideum*; Ryan & Semlitsch, 1998). Thus, very different patterns of growth and development may be associated with the expression of paedomorphosis within and between species (Whiteman, 1994). Among species that occur in stable aquatic

habitats, paedomorphosis is a fixed trait with little or no incidence of metamorphosis. Within species that use less permanent bodies of water for breeding, individuals may reproduce initially as paedomorphs but undergo metamorphosis in subsequent years (Denoël et al., 2007). The different patterns of growth and development observed among paedomorphs yield different adult morphologies, and this suggests different mechanistic bases for the evolution of paedomorphosis within and among salamander families. However, exactly how patterns of development and morphology map onto mechanisms that block TH signaling and metamorphosis, or affect the rate of gonadal development, is essentially unknown. Without such mechanistic information, it is not possible to distinguish among examples of paedomorphosis that evolve convergently among lineages, which clearly has occurred during salamander phylogenesis (Shaffer & Voss, 1996; Weins & Hoverman, 2008). Thus, all terms that *describe* the evolutionary origin or developmental expression of paedomorphic salamanders are potentially useful metaphors, but if they are not based upon mechanistic insight and genetically based models, they are not likely to resolve outstanding questions concerning salamander life history variation and life cycle evolution.

It is interesting to consider why paedomorphosis is unique to salamanders and not other amphibians. In as much as paedomorphosis evolves as a prolongation of the larval period, it is notable that salamanders with biphasic life cycles typically have longer larval periods than caecilians or anurans (Duellman & Trueb, 1986). In biphasic salamander species, metamorphosis may occur within the same season or year that eggs are laid or larvae may overwinter in permanent habitats and metamorphose after one or more additional years of development (e.g., Beachy, 1995; Castanet, Francillon-Vieillot, & Bruce, 1996; Voss, Prudic, Oliver, & Shaffer, 2003). Although there are examples of anurans with multiyear larval periods, salamander larval periods are typically longer. So, there are fundamental differences between salamanders and other amphibians; salamanders have greater evolutionary potential to protract (and contract in cases of direct development) the length of the larval period. This greater evolutionary potential is associated with at least five aspects of salamander development and life history. First, salamanders exhibit slow rates of growth and development. Some anurans can complete metamorphosis in just a couple of weeks, while the fastest developing salamander larvae require more than a month, and typically several months (Duellman & Trueb, 1986). In general, salamander metamorphosis is developmentally less radical and more protracted than that of anurans. For example, salamander forelimb development does not

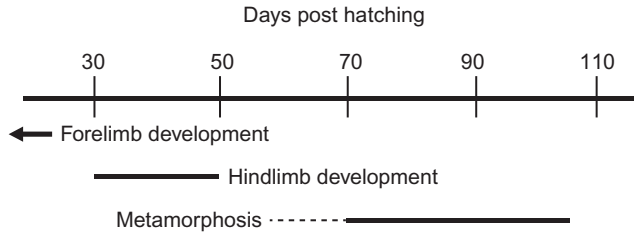

Figure 8.2 Timeline showing timing of fore and hindlimb development and the metamorphic period of laboratory reared *A. t. tigrinum*. The dashed line indicates variation among individuals in presenting early signatures of anatomical metamorphosis (bulging eyes, reduced tail fins). In the anuran *X. laevis*, hindlimb and forelimb development initiates 2 weeks after hatching. Hindlimbs complete development prior to metamorphosis, while forelimbs emerge during metamorphosis.

coincide with other morphological metamorphic changes, as it does in anurans (Fig. 8.2). Instead, it occurs shortly after hatching and before gills and tailfins are resorbed at metamorphosis. This suggests either a decoupling of forelimb development from metamorphosis or an incredibly gradual metamorphosis in salamanders that essentially spans the entirety of the larval period (Duellman & Trueb, 1986). Second, aspects of TH signaling may differ between salamanders and other amphibians. While it is clear that TH is required for salamander metamorphosis, *Ambystoma mexicanum* tolerates levels of TH that cause abnormal development and mortality in some anurans (Page et al., 2008). Also, several orthologous genes, including *thyroid hormone receptor beta* (*thrb*), do not show the same pattern of expression between TH-induced *A. mexicanum* and metamorphosing anurans (Page et al., 2007, 2008). Third, salamanders often utilize low temperature habitats, either because they occur at relatively high elevations or latitudes (Bizer, 1978; Sexton & Bizer, 1978). Low temperature slows growth and development, depresses activity of neurons and glands that regulate metamorphosis, and reduces the responsiveness of tissues to TH (Moriya, 1983a, 1983b; Uhlenhuth, 1919). Fourth, salamander larvae present the same body design as adults. Thus, unlike anuran tadpoles that must reorganize the body cavity at metamorphosis to allow space for gonads to develop, salamanders can potentially allocate resources to developing gonads and associated fat bodies during the larval period. Fifth, salamander larvae are carnivorous like adults, and thus unlike herbivorous anuran tadpoles, do not rely upon seasonal, primary productivity of ponds for food. Many anuran species have tadpoles that are highly specialized for rapid development, utilizing seasonally abundant resources in ephemeral ponds that select for finite

larval periods (Wilbur, 1980). Multiyear larval periods are simply not possible within the context of anuran life history strategies that depend upon seasonally limiting resources. In contrast, salamander larvae present greater developmental flexibility to utilize more permanent aquatic habitats by extending the length of the larval period, indefinitely in the case of some paedomorphic species.

2. PAEDOMORPHOSIS AND SALAMANDER LIFE HISTORY VARIATION

Salamander life histories can be broadly classified into three categories: biphasic, paedomorphic, and facultative (Table 8.1). These are simply categories of convenience and do not adequately treat the continuum of life histories represented by each class, and the possibility of different genetic, developmental, and physiological bases within classes. Species with biphasic life histories almost invariably undergo a metamorphosis. This life history strategy is viewed as an adaptation to exploit transient opportunities for larval

Table 8.1 General characteristics of metamorphic, paedomorphic, and facultative salamander life histories

Metamorphic	Paedomorphic	Facultative
Associated with ephemeral aquatic habitats[a]	Associated with stable aquatic habitats[b]	Associated with landscapes with ephemeral and stable aquatic habitats[c]
Individuals almost invariably metamorphose in nature[a]	Individuals rarely/never metamorphose in nature[b]	Populations often vary in the proportion of individuals that metamorphose or exhibit paedomorphosis[c]
Generally reach sexual maturity after metamorphosis[c,d]	Reach sexual maturity while remaining in larval aquatic habitat[d,e]	Timing of sexual maturity may vary between metamorphic and paedomorphic individuals of the same population (but not always)[f–h]
Thyroid hormones cause morphological changes (e.g., gill reduction, larval to adult skin changes, tail fin reduction, etc.)[i]	Individuals may or may not respond to thyroid hormone with morphological signs of metamorphosis[i]	Thyroid hormones cause morphological changes[j,k]

Table 8.1 General characteristics of metamorphic, paedomorphic, and facultative salamander life histories—cont'd

Metamorphic	Paedomorphic	Facultative
Species may respond to stressful environmental factors with precocious metamorphosis[l]	In rare cases, stressful environmental factors may cause some species to metamorphose (e.g., *A. mexicanum*)[m,n]	Environmental factors affect the probability of an individual to express metamorphosis or paedomorphosis[b,o,p]

[a]Wilbur (1980).
[b]Sprules (1974).
[c]Denoel, Joly, and Whiteman (2005).
[d]Duellman and Trueb (1986).
[e]Armstrong, Duhon, and Malacinski (1989).
[f]Scott (1993).
[g]Ryan and Semlitsch (1998).
[h]Denoel and Joly (2000).
[i]Bruce (2003).
[j]Snyder (1956).
[k]Voss, Kump, Walker, Shaffer, and Voss (2012).
[l]Wilbur and Collins (1973).
[m]Smith (1969).
[n]Voss (unpublished observation).
[o]Eagleson and McKeown (1980).
[p]Semlitsch (1987).

growth and is often found where aquatic habitats are ephemeral (Wilbur, 1980; Wilbur & Collins, 1973). At the opposite extreme are salamanders that almost invariably remain paedomorphic until they die as aquatic adults. Paedomorphic salamanders are associated with isolated and stable aquatic habitats, such as large closed-basin lakes, spring-fed lakes, caldera lakes, and river systems (Shaffer, 1984; Sprules, 1974). In facultative species, metamorphs and paedomorphs are observed at varying frequencies within the same population. In such species, it is thought that the ability to express paedomorphosis and metamorphosis is advantageous when a landscape contains a mixture of ephemeral and stable aquatic habitats (Denoel et al., 2005; Semlitsch, Harris, & Wilbur, 1990; Sexton & Bizer, 1978; Whiteman, 1994; Wilbur, 1980; Wilbur & Collins, 1973). Facultative life histories are sometimes associated with aquatic habitats that may only be permanent for a single year of reproduction; the fitness advantage must be considerable because it is not uncommon to observe a paedomorphic brood in a cattle-watering tank on an arid landscape with no permanent ponds (Randal Voss, personal observation). When facultative species lay eggs in habitats that decline in quality during the larval period, metamorphosis is initiated in all or some proportion of individuals in the population (Semlitsch, 1987; Semlitsch et al., 1990).

Whereas metamorphic and facultative taxa are capable of dispersal among aquatic habitats, obligatorily paedomorphic taxa are confined to isolated bodies of water. This suggests that biphasic or facultative ancestors colonized aquatic habitats and ecological conditions (e.g., permanent aquatic habitat and/or arid terrestrial conditions) selected for paedomorphic life histories. The expression of paedomorphic life histories is known to affect population structure and may affect the probability of speciation (Shaffer, 1984; Shaffer & Breeden, 1989). The fitness benefits of paedomorphosis are an earlier time to first reproduction, more than one breeding event per year, larger clutch size, and a higher probability of mating success (Krenz & Server, 1995; Ryan & Semlitsch, 1998; Scott, 1993). It is important to note that paedomorphosis is not a pathology or default life history strategy—a failure to undergo metamorphosis. Paedomorphic species are highly adapted to their aquatic habitats. For example, *Ambystoma dumerilii* has secondarily evolved webbed-feet and extra gill filaments that develop at the time metamorphosis occurs in related metamorphic species (Fig. 8.3). Thus, evolution of paedomorphosis may release tissues from the constraint of TH remodeling, allowing new functions to evolve that better adapt adults to aquatic habitats. The combination of selection and genetic drift acting on isolated paedomorphic lineages may explain rapid divergence of morphology and gene expression within some species groups (Page et al., 2010; Shaffer & Voss, 1996).

Paedomorphic life history strategies are widespread among salamanders, occurring in nine out of 10 families with a total of 57 species. Studies show that paedomorphic salamanders have evolved multiple times in different lineages (Denoel et al., 2005; Shaffer & Voss, 1996; Weins & Hoverman, 2008). Four salamander families (Amphiumidae, Sirenidae, Proteidae, and Cryptobranchidae) are comprised entirely of paedomorphic species, having no extant biphasic species. Some salamanders, such as species in the families Cryptobranchidae and Amphiumidae, are said to undergo an incomplete metamorphosis—meaning they undergo some anatomical changes, but not others, and remain aquatic throughout their lives. For example, adult Hellbenders (*Cryptobranchus alleganiensis*) lose their larval external gills, but do not develop eyelids and retain a single gill-slit that appears as a circular opening on the neck (Larson, Weisrock, & Kozak, 2003). The most species-rich salamander family, Plethodontidae, includes many biphasic and direct developing species, with the Tribe Hemidactyliini (Subfamily Plethodontinae) containing numerous paedomorphic species that are cave dwelling and exhibit reduced pigmentation and vision (Larson et al., 2003). Perhaps the best-studied salamander families with respect to life history variation are

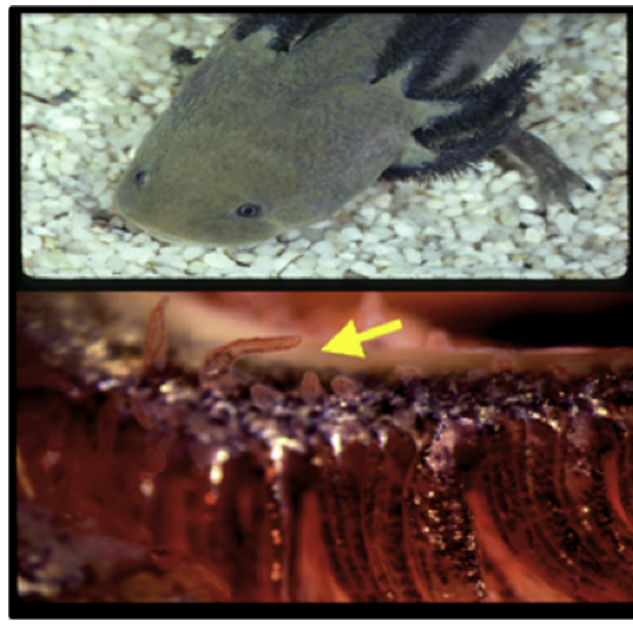

Figure 8.3 *Ambystoma dumerilii* is a paedomorph that has secondarily evolved webbed-feet and extra filaments that develop on the dorsal surfaces of each gill. The bottom panel is a magnified image of the dorsal gill surface. The arrow indicates dorsal filaments (<1.0 mm in length) that are sprouting on the gill surface. These extra fibers form late in the larval period, at the time metamorphosis normally occurs in related metamorphic species. *The A. dumerilii picture was taken by Brad Shaffer.*

Salamandridae and Ambystomatidae. Studies of paedomorphic and facultative species within these families have better elucidated environmental and broad-sense genetic correlates of metamorph and paedomorph expression, and these species continue to provide exceptional models for investigating ecological aspects of life history evolution (Denoel, Ivanovic, Dzukic, & Kalezic, 2009; Denoel et al., 2005; Doyle & Whitemann, 2008; Takahashi & Parris, 2008; Whiteman et al., 2012).

3. HORMONAL BASIS OF SALAMANDER METAMORPHOSIS

Biphasic, paedomorphic, and facultative life histories begin the same way, with embryonic and larval development occurring in an aquatic habitat. The primary difference is when and if metamorphosis occurs. Although

not as dramatic as metamorphosis in anurans, multiple tissues undergo hormonally regulated changes in cellular composition, biochemistry, and morphology during salamander metamorphosis (Dodd & Dodd, 1976). These changes include degeneration of external gills and tailfins; remodeling of skeletal elements and integument; shortening of the intestine; modification of pigmentation; muscle degeneration and differentiation; and changes in nitrogen excretion, urogenital function, and oxygen transport (Duellman & Trueb, 1986). Also, new structures arise *de novo*, including eyelids and skin glands. All of these changes adapt aquatic larvae for survival and reproduction as adults in more terrestrial environments.

Although salamanders have a more protracted and gradual metamorphosis than anurans, TH is the primary metamorphic hormone in both amphibian groups. Serum TH in the form of tetraiodothyroine (T4) is low during embryonic and early larval development, but as larvae approach metamorphosis, T4 levels increase (Norman, Carr, & Norris, 1987). This pattern of hormone secretion is not unlike that observed for other examples of hormonally regulated, postembryonic development (e.g., insect and fish metamorphosis; Gilbert, Tata, & Atkinson, 1996; Laudet, 2011; Power et al., 2001). The rise in T4 during amphibian larval development is associated with maturation and stimulation of brain regions that regulate thyroid activity, in response to extrinsic and intrinsic cues. These brain regions include the hypothalamus and pituitary gland. Increasing levels of T4 during larval development stimulate corticotropin-releasing hormone (CRH) release from modified nerve endings within the median eminence. CRH travels through capillaries to the anterior pituitary where it stimulates thyrotropes to release thyrotropin (TSH) into the blood stream. TSH in turn stimulates the thyroid gland to release T4. The TSH-releasing factor in amphibians is CRH, which also stimulates the release of corticotropin (ACTH) and subsequently, glucocorticoids (Denver & Licht, 1989). The potent, synergistic action of corticoids and TH in promoting anuran and salamander metamorphosis clearly implicates the hypothalamic-pituitary-thyroid (HPT) and HP-interrenal (HPI) axes in the physiological regulation of metamorphosis (Buscaglia, Leloup, & De Luze, 1985; Dodd & Dodd, 1976; Galton, 1991, 1992; Gancedo et al., 1992; Hayes, Chan, & Licht, 1993; Kuhn, De Groef, Van der Geyten, & Darras, 2005). Exactly how T4 levels initially increase at appropriate times during amphibian development to trigger the onset of metamorphosis, and sustain T4 at high levels during metamorphosis, is not fully understood (Manzon & Denver, 2004).

Peripherally, the availability and activity of TH is precisely regulated within cells. In mammals, TH is transported into target cells via TH transporters, which actively and preferentially import the hormone across the plasma membrane (Hennemann et al., 2001). TH transporters have been studied in *X. laevis* (Connors, Korte, Anderson, & Degitz, 2010; Ritchie et al., 2003), but there have been no studies using salamanders. Deiodinases have received considerable attention because they directly activate or inactivate intracellular forms of TH in amphibians (Buscaglia et al., 1985; Galton, 1991; Kuhn et al., 2005). The low activity T4 form of TH that is delivered to cells is converted intracellularly by deiodinase-type 2 (DIO2) into highly active 3,5,3′-triiodothyronine (T3). A second deiodinase, DIO3, inactivates intracellular T3. Although T4 may participate in cellular signaling (Caria, Dratman, Kow, Mameli, & Pavlides, 2008), T3 is the primary TH signaling molecule in anurans (Denver et al., 2002) and presumably this is also true for salamanders, acting through nuclear receptors (*thra*, *thrb*) that function as transcription factors. The higher activity of T3 is associated with a higher affinity for TH receptor (TR) binding. The binding of T3 activates transcriptional regulatory networks in cell-specific manners (Shi, 2000). TH is also known to cause cellular changes via nongenomic mechanisms, including membrane receptors, transporter molecules, and cytoplasmic receptors that elicit changes in cells through signaling pathways (Caria et al., 2008; Cheng, Leonard, & Davis, 2010; Davis, Leonard, & Davis, 2008; Furuya, Lu, Guigon, & Cheng, 2009). The effects of these nongenomic mechanisms of TH action and their role in amphibian metamorphosis have yet to be studied in detail, however, Buchholz, Tomita, Fu, Paul, and Shi (2004) used transgenic analysis to show that TR is sufficient to mediate TH signaling during metamorphosis in *X. laevis*. Regulation of deiodinases, T4/T3 concentrations, and developmental expression of TRs help explain how a single hormone can coordinate responses among different cell types, and more generally regulate the temporal sequence of remodeling events during amphibian metamorphosis (Brown & Cai, 2007).

4. HORMONAL BASIS OF PAEDOMORPHOSIS

Only a year after, Gudernatsch (1912) established TH as the anuran metamorphic hormone, TH was shown by Laufberger to induce metamorphosis in *A. mexicanum* (Mexican axolotl) (reviewed by Huxley & Hogben, 1922). Over the next few decades, the responsiveness of many different paedomorphic salamanders to TH was investigated. Studies showed that

morphologically similar paedomorphic salamanders (with external gills or gill slits, no eyelids, larval pigmentation, etc.) responded differently to TH. Members of the family Proteidae, such as the American mudpuppy (*Necturus maculosus*), are known to be refractory to TH, such that treating them with exogenous or injected TH does not induce morphological metamorphosis (Swingle, 1922). Larsen (1968) proposed that peripheral tissues of *N. maculosus* had lost sensitivity to TH, given that it exhibits a morphologically normal thyroid gland that had been tested for functionality via transplant experiments (Charipper, 1929; Grant, 1930; Swingle, 1922). This prompted investigations into the functionality of the animal's deiodinase activity and TR expression. Galton (1985) found that deiodinase activity capable of converting T4 to T3 was present within *N. maculosus*, and Safi et al. (2006) showed that *N. maculosus* expresses functional TRs that bind to DNA and activate transcription in response to TH treatment, including transcriptional changes of classical TR target genes like *stromelysin 3* (*mmp11*) and *thrb* (Safi et al., 2006; Vlaeminck-Guillem et al., 2006). Additionally, *in situ* hybridization has been used to show expression of *thra* and *thrb* in central nervous system, epithelia of the digestive tract, myocardium, and skeletal muscle (Vlaeminck-Guillem et al., 2006). These studies suggest that the paedomorphic phenotype exhibited by *N. maculosus* may be due to the loss of key TH response genes needed to induce morphological changes, even though juveniles experience a postembryonic period of high TH sensitivity. Apparently, individuals undergo natural transcriptional and biochemical changes during a "cryptic metamorphosis" that does not result in gross morphological alterations (Laudet, 2011; Vlaeminck-Guillem et al., 2006). Species of the families Cryptobranchidae and Amphiumidae are responsive to THs in some respects, but not others. They often reabsorb external gills and develop adult characteristics of some features, but do not develop eyelids and retain gill slits as mature adults, indicating that some metamorphic processes may have been decoupled from TH regulation (Larson et al., 2003). When species of these families are treated with high doses of TH, often the only result is skin shedding, a trait that is regulated by TH in metamorphic species, though otherwise the individual continues to exhibit paedomorphic characteristics (Dent, 1968; Fox, 1984). It was originally thought that the Texas blind salamander (*Eurycea rathbuni*, formerly assigned to the genus *Typhlomolge*) was the only known vertebrate to lack a thyroid gland (Emerson, 1905). However, Gorbman (1957) later showed the presence of a thyroid gland in *E. rathbuni* and Dundee (1957) showed that an individual of this species underwent some morphological

changes (gill and tail reabsorption) in response to T4, but other larval features were resistant (histological sections of skin revealed only larval characteristics). However, related paedomorphic species (*E. tynerensis* and *E. neotenes*) complete metamorphosis after T4 treatment in less than 2 weeks (Kezer, 1952). Paedomorphic species of *Ambystoma* also complete metamorphosis when treated with T4 but show species-specific variation in metamorphic timing (Voss et al., 2012). Collectively, these studies establish that paedomorphosis is associated with TH regulation and tissue responsiveness, and these characteristics vary among closely and distantly related species.

Based on results of early TH induction experiments, it was proposed that paedomorphic species evolve via several mechanisms, including disruption of peripheral and central mechanisms of TH regulation (Dent, 1968; Kezer, 1952). Species that were shown to be refractory to TH but had active thyroid glands were presumed to be deficient in peripheral mechanisms that receive and transduce the T4 signal, such as TRs. Species that responded to high levels of TH but did not apparently metamorphose in nature were assumed to be deficient in some aspect of central regulation within the hypothalamus or pituitary. Such a deficiency would normally prevent T4 release but could artificially be over-ridden in the lab by T4 treatment. It also seems likely that central regulatory mechanisms have been altered in the evolution of facultative species from biphasic ancestors. This is because the brain and pituitary regulate appropriate physiological and developmental responses in response to environmental cues. In the case of facultative paedomorphosis, the HPT axis is not sufficiently activated if cues favor a paedomorphic life history, or environmental conditions suppress hormone activity, or if larval development and maturation is delayed (Whiteman, 1994). Several environmental conditions that are associated with habitat quality affect the probability that an individual will express paedomorphosis, including low density of conspecifics (Harris, 1987; Semlitsch, 1987), food availability (Denoel & Poncin, 2001; Ryan & Semlitsch, 2003; Semlitsch, 1987), pond permanence (Semlitsch, 1987; Semlitsch et al., 1990), and low temperature (Eagleson & McKeown, 1980; Snyder, 1956; Sprules, 1974).

5. PAEDOMORPHOSIS IN THE MEXICAN AXOLOTL

The exemplar of all paedomorphic salamanders is the Mexican axolotl (*A. mexicanum*). The Mexican axolotl is native to the Xochimilco lake system, near present-day Mexico City. Whereas historical populations were sufficiently large to provide an important food source to Indians for

thousands of years (Smith, 1989), the current *A. mexicanum* population at Xochimilco is on the verge of extinction (Graue, 1998; Zambrano, Vega, Herrera, Prado, & Reynoso, 2007). Smith (1989) provides an enjoyable historical account of the original collection of *A. mexicanum* from Xochimilco in 1863. Six *A. mexicanum* were transferred to Paris where Dumeril (1870) subsequently reported that individuals reproduced in an aquatic, larval state and that some of the resulting offspring underwent a metamorphosis. Smith (1989) suggests that some of the original stock that arrived in Paris included closely related members of the Tiger salamander species complex, that are capable of expressing a paedomorphic or metamorphic life history. However, it seems likely that domestication altered the penetrance for expressing paedomorphosis and the original axolotl stock was pure *A. mexicanum* that maintained a higher propensity to express metamorphosis in nature (Voss & Shaffer, 2000). Even though metamorphic forms have been culled from the Ambystoma Genetic Stock Center axolotl collection for decades, the frequency of spontaneous metamorphosis is ~1–2%, with a 10% frequency observed if *A. mexicanum* experience stressful conditions (Randal Voss, unpublished data).

The simplest explanation for paedomorphosis in *A. mexicanum* is a faulty thyroid gland. However, the thyroid gland is functional; it can synthesize T4 and release T4 after TSH stimulation (Prahlad, 1968; Taurog, Oliver, Eskay, Porter, & Mckenzie, 1974). Numerous studies, including our own (Page et al., 2007, 2008), have demonstrated that individuals can be stimulated to undergo morphological metamorphosis by administering T4. This suggests that intracellular receptors and deiodinase enzymes are functional. Putative T3 receptors have been identified in red blood cells (Galton, 1991) and TR function has been shown *in vitro* using mammalian cells (Safi et al., 2004). Also, the activity of deiodinases has been demonstrated (Darras et al., 2002; Galton, 1991). Blount (1950) showed that metamorphosis is inhibited if the pituitary of *A. mexicanum* is transplanted into the metamorph *A. tigrinum*, while the reciprocal transplantation experiment resulted in metamorphosis. Tassava (1969) pointed out that these grafts were probably not precise enough to rule out a hypothalamic contribution. TSH is present in the *A. mexicanum* pituitary (Taurog et al., 1974) at a quantity sufficient for inducing metamorphosis (Darras & Kuhn, 1983). Thus, the pituitary is capable of producing sufficient functional TSH for metamorphosis, but for some reason, it is not released. There are at least four explanations for this observation in *A. mexicanum*: (1) The pituitary does not receive appropriate stimulation from the hypothalamus, (2) the pituitary is insensitive to hypothalamic stimulation, (3) the pituitary is defective in releasing TSH, and (4) the pituitary is functional but stimulated to release TSH at the wrong

time during ontogeny (Rosenkilde & Ussing, 1996). Galton (1992) suggested that T4 levels might never eclipse a threshold during development to stimulate brain regions to increase activity of the thyroid gland. Kuhn et al. (2005) similarly proposed that brain T3 availability is the limiting factor for metamorphic onset. They found that DIO2 activity in the brain, which is required to generate T3, requires appropriate doses of corticoids and T4. Thus, stimulation of both the HPA and HPI axes is required for metamorphosis, and in fact, it is possible to induce metamorphosis in *A. mexicanum* if submetamorphic levels of T4 and corticoids are administered together. Because injection of amniote CRH stimulates corticotropin but not thyrotropin release, Kuhn et al. (2005) implicated the thyrotroph CRH receptor as a likely candidate gene for paedomorphosis in *A. mexicanum*. However, researchers have demonstrated T4 increases after stimulating hypothalamic regions in neotenic tiger salamanders (*A. tigrinum*) and *A. mexicanum*; this could seemingly only occur if downstream thyrotrophin signaling pathways are functional (Norris & Gern, 1976; Rosenkilde & Ussing, 1996).

It is also possible that a recently discovered TSH signaling pathway may be implicated in salamander paedomorphosis. Studies of neuroendocrine regulation of amniote reproductive cycles showed that TSH from the *pars tuberalis* (PT) of the anterior pituitary gland is released to signal DIO2 transcription in the hypothalamus (Hanon et al., 2008, Nakao et al., 2008). This increases local T3 concentrations and thus stimulates canonical TH signaling pathways. Surprisingly, these studies show that the flow of information may be bidirectional between the pituitary and hypothalamus. If a similar PT signaling pathway is necessary for metamorphosis, this pathway would not be activated in *A. mexicanum* because of a block in TSH release. This might also explain why *A. mexicanum* does not show strong seasonality in breeding and can breed multiple times a year. Whether paedomorphosis involves a PT signaling mechanism or a classical TSH signaling mechanism, the metamorphic block in *A. mexicanum* is likely associated with hypothalamus and pituitary function (Kuhn et al., 2005; Laudet, 2011; Norris et al., 1973; Prahlad & DeLanney, 1965; Rosenkilde & Ussing, 1996).

6. GENOMIC ANALYSIS OF SALAMANDER METAMORPHOSIS AND PAEDOMORPHOSIS

Most mechanistic studies of metamorphosis and paedomorphosis have focused on physiology and to a lesser extent biochemistry. Recently, microarrays developed for *A. mexicanum* have been used to characterize global transcriptional responses of metamorphic and paedomorphic salamanders.

These studies have focused on the skin and brain, the former because it is a peripheral target tissue of TH and much is known about anatomical changes that occur during metamorphosis (Fahrmann, 1971a, 1971b, 1971c; Fox, 1983; Page, Monaghan, Walker, & Voss, 2009). The brain has also been targeted because it is where HPT and HPI axes are centrally regulated. We review three important findings from these studies.

T4 precisely activates metamorphic transcriptional programs in a concentration-dependent manner. Page et al. (2008) varied T4 concentration by an order of magnitude and examined transcriptional patterns in *A. mexicanum* skin after 0, 2, 12, and 28 days of T4 treatment. Individuals exposed to 50 nM T4 initiated metamorphosis approximately 1 week prior to those in 5 nM, however, the sequence of transcriptional and morphological changes during metamorphosis, and the length of the metamorphic period did not differ (Fig. 8.4A). Over 1000 genes were identified as differently expressed in both treatments, showing the same directional trends, but the patterns were temporally shifted by T4 concentration. The study showed that the timing of metamorphic onset is T4 concentration dependent, and surprisingly, high T4 concentration did not disassociate subsequent transcriptional programs. It remains to be determined if the T4 concentration effect in *A. mexicanum* is manifested at the level of target cells, the HPT axis, or possibly both (Rosenkilde & Ussing, 1996).

Patterns of gene expression are complex, reflecting tissue remodeling, changes in cellular metabolism, and loss and gain of larval and adult cell types. One of the advantages in working with *A. mexicanum* is that they present a natural, hypothyroid condition throughout life (Huggins et al., 2012). Thus, transcriptional programs can be activated precisely at juvenile or adult stages of the life cycle. When T4 is administered, thousands of genes show significant changes in transcript abundance. In a very general sense, these genes show four temporal gene expression patterns: they increase or decrease in abundance, or they transiently increase or decrease during metamorphosis (Fig. 8.4B). Linear, quadratic, and piece-wise regression models have been used to identify groups of genes that similarly increase or decrease in abundance as a function of days of T4 treatment. The earliest gene expression changes precede tissue-remodeling events and are associated with cells that are specific to larvae or cells that differentiate to form adult tissues. For example, after 2 days of T4 treatment, genes that are specifically expressed in apical and Leydig cells of the larval skin show decreases in transcript abundances (Page et al., 2007, 2008, 2009). At this time, apical and Leydig cell numbers are normal for larval skin and there is no histological evidence of remodeling. However, after 12 days of T4

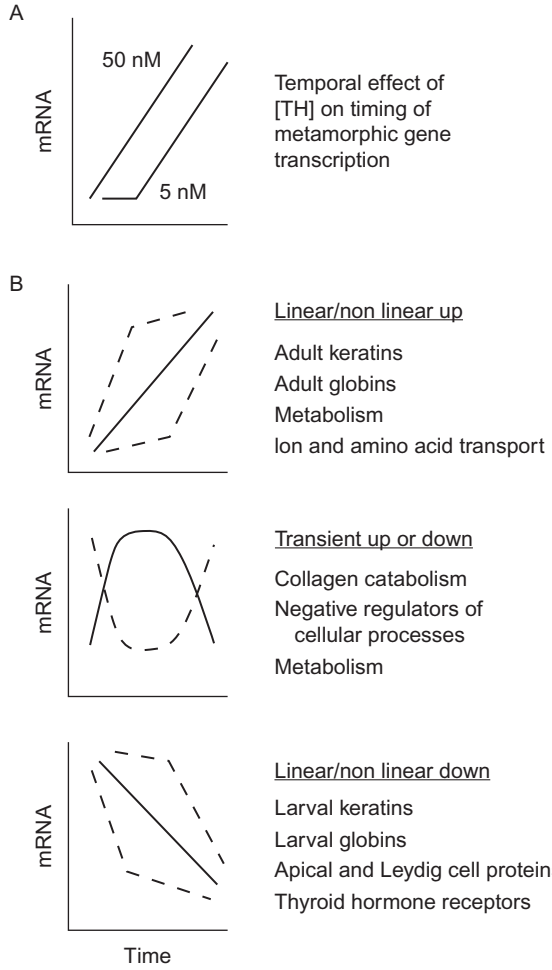

Figure 8.4 (A) Representative expression profile for a gene that shows a linear increase in mRNA abundance after TH induction of *A. mexicanum* using 5 or 50 nM T4. (B) Four general types of gene expression response observed after TH induction of metamorphosis in *A. mexicanum*. See text for details.

treatment, apical cells are no longer present in histological sections and Leydig cells are significantly reduced in number (Page et al., 2009); presumably, both cell types undergo TH-induced, programmed cell death, as has been demonstrated for apical cells in metamorphosing anurans (Schreiber & Brown, 2002). As larval cells and their gene expression products decrease in abundance during metamorphosis, TH induces the proliferation and differentiation of adult progenitor cells. As a result, transcripts associated with adult cells increase in

abundance. The most dramatic changes are seen for basal keratinocytes that proliferate and differentiate during metamorphosis and transcribe adult specific keratins that generate a more cornified epithelial layer to limit desiccation during the adult phase. Genes that transiently increase in abundance code for matrix-remodeling proteins (e.g., MMPs) and cellular processes like metabolism, transcription, and translation, while genes that decrease in abundance include negative regulators of cellular processes (Page et al., 2008). These gene expression patterns illuminate tissue remodeling and macromolecular biosynthetic processes that occur during metamorphic climax.

Although some genes are expressed similarly between metamorphic and paedomorphic individuals during larval development, the majority of the differentially expressed genes are unique. For example, brain transcription has been compared between *A. mexicanum* and *A. t. tigrinum* individuals during larval development and the period spanning the metamorphic period of the later species (Page et al., 2010). As *A. t. tigrinum* individuals near the onset of anatomical metamorphosis, more gene expression differences are observed between larvae of these species. Presumably, many of these expression differences trace to increasing levels of glucocorticoids and THs in *A. t. tigrinum* individuals as they initiate early metamorphic changes. In support of this idea, a glucocorticoid receptor (*nr3c2*) showed significantly higher expression in *A. t. tigrinum*. Also, some genes that were more highly expressed during natural metamorphosis in *A. t. tigrinum* were also similarly upregulated in *A. mexicanum* brains when individuals were induced with T4 (Huggins et al., 2012). However, hundreds of genes were expressed more highly in *A. t. tigrinum* than *A. mexicanum* throughout the larval period. At this point, it is not clear if all of these differences are associated with metamorphic and paedomorphic life histories; however, the observed systematic bias in transcription suggests more than species-specific divergence of gene expression.

7. GENETIC ANALYSIS OF PAEDOMORPHOSIS

The idea that paedomorphic salamanders simply need a dose of TH to induce the second half of the ancestral ontogeny influenced the way paedomorphic life histories were thought to originate and evolve. For example, Goldschmidt (1940) cited *A. mexicanum* as the "hopeful monster" (Page et al., 2010), an example of evolution waiting around for the right macromutation—simply block a single physiological step in TH regulation and instantly a novel form is originated. The idea that paedomorphosis results from a simple mechanistic change gained further support when genetic

studies showed that metamorphic and paedomorphic forms could be segregated according to Mendelian expectations. In particular, Humphrey (1967) showed that when *A. mexicanum*/*A. t. tigrinum* hybrids were backcrossed to *A. mexicanum*, there is 1:1 segregation of phenotypes. Later, Tompkins (1978) and Gould (1981) argued from Humphrey's single cross that *A. mexicanum* was an example of single gene, macromutational evolution. To further test the single gene hypothesis, Voss (1995) crossed domesticated *A. mexicanum* and *A. t. tigrinum* to create F_1 hybrids and like Humphrey (1967), backcrossed these to *A. mexicanum*. The ratios of metamorphs and paedomorphs arising from two relatively large backcrosses were consistent with single locus control, thus supporting the classical idea of a single mutation underlying the evolution of paedomorphosis. In these crosses, the *A. t. tigrinum* and *A. mexicanum* alleles were dominant and recessive in effect, respectively. Subsequently, DNA was isolated from individuals of these crosses, amplified fragment length polymorphisms (AFPLs) were typed, and a genetic linkage map was constructed. This map was used to roughly locate the position of the major gene (*met1*) for paedomorphosis (Voss & Shaffer, 1996). To determine if *met1* arose independently in the domesticated stock, the backcross design was repeated using *A. mexicanum* collected from Lake Xochimilco (Voss & Shaffer, 2000). The segregation of phenotypes did not fit a single gene model, and the segregation of AFLPs marking the *met1* region exhibited segregation distortion, suggesting an epistatic effect between *met1* and other loci on viability. Still, *met1* genotypes did associate with metamorphic and paedomorphic phenotypes, indicating a difference in genetic background between wild-caught *A. mexicanum* and the domesticated stock.

Voss and Smith (2005) made additional *A. mexicanum*/*A. t. tigrinum* backcrosses and reared over 500 offspring to rigorously quantify the genetic effect of *met1* from wild-caught *A. mexicanum*. These crosses showed that *met1* not only explained discrete variation in the expression of metamorphosis and paedomorphosis but also continuous variation in metamorphic timing. Approximately 20% of the individuals that inherited two *met1* alleles from wild *A. mexicanum* were paedomorphic. The remainder of these homozygotes delayed metamorphic timing by an average of 36 days, relative to heterozygotes. These results showed that *met1* determines the expression of metamorphosis and paedomorphosis by altering metamorphic timing. Within the context of hybrid crosses, alleles from metamorphic and paedomorphic species decrease and delay the time to metamorphosis, respectively. Variation in the number of paedomorphic individuals generated from wild-caught versus domesticated *A. mexicanum* crosses reflect differences

in genetic background. Here, we define genetic background as the summative effect of loci whose independent effects are too small to identify and quantify statistically. Within the genetic background of the domesticated stock, which has undergone strong artificial selection for paedomorphosis, the penetrance of *met1* is greater, and this results in a higher proportion of paedomorphic individuals in hybrid crosses (Voss & Smith, 2005).

8. THE LINK BETWEEN *met1* AND TH REGULATION OF METAMORPHOSIS

The link between *met1* and TH was recently established by combining quantitative trait locus (QTL) analysis with TH induction. Recognizing that paedomorphic species show variation in time to complete metamorphosis when treated with TH, Voss et al. (2012) crossed two paedomorphic species (*A. mexicanum* and *A. andersoni*) and then performed a backcross to segregate TH response alleles among second generation offspring. At 120 days postfertilization (dpf), backcross offspring were treated with 2.5 nM T4 and then scored for time to complete metamorphosis. Essentially, all offspring initiated and completed metamorphosis over an ~160 day interval of time. A QTL screen identified the genomic position of *met1* and two additional QTL (*met2*, *met3*), each explaining approximately 10% of the variation in metamorphic timing; the effects of all alleles were additive with *A. andersoni* alleles decreasing the time to metamorphosis. On average, individuals that inherited *met1-3* alleles from *A. andersoni* metamorphosed earlier, just as did hybrid *A. mexicanum* that inherited *met1* alleles from metamorphic *A. t. tigrinum* (Voss & Smith, 2005). The decrease in metamorphic timing was approximately 19 days for *A. andersoni met1* versus 36 days for *A. t. tigrinum met1*. These results demonstrate that variation in metamorphic timing is determined in part by alleles that segregate at TH-responsive QTL, and alleles from paedomorphic species may vary in effect.

In natural populations of amphibians, variation in metamorphic timing can affect the values of other life history traits. Life history theories predict that individuals should metamorphose early if conditions for larval growth are poor, but delay metamorphosis and attain large larval body sizes if growth conditions are optimal (Gould, 1977; Wilbur & Collins, 1973). This is because larger body sizes at metamorphosis translate into increased reproductive success in the adult phase (Semlitsch et al., 1988). From a physiological perspective, metamorphic timing and body size are expected to covary as a function of resource allocation. From an evolutionary perspective,

metamorphic timing and body size are expected to covary and coevolve if they share a common genetic basis. This prediction holds in the case of *met2*. In the *A. andersoni*/*A. mexicanum* backcross study described above, *met2* not only explained significant variation in metamorphic timing but it also explained significant variation in adult body size (Voss et al., 2012). Individuals that delayed metamorphosis significantly increased body length at 300 dpf and weight at 400 dpf, and the effect was approximately equivalent for sexually dimorphic males and females. Overall, the genetic results reviewed here show that TH-responsive QTL accounts for variation in metamorphic timing, expression of metamorphosis versus paedomorphosis, and adult fitness traits.

9. WHAT GENES MAP TO METAMORPHIC TIMING QTL?

The *A. mexicanum* genome is in early stages of genome sequencing and thus comparative mapping has been used to more finely resolve the positions of *met1-3* and identify candidate genes. The most promising candidate that has been identified is *pou1f1*, a transcription factor associated with combined pituitary hormone deficiency (CPHD) in humans. *pou1f1* is predicted to map to the position of *met3* on linkage group (LG) 7. CPHD is associated with deficiencies in the secretion of pituitary hormones that regulate growth and development, but not hormones associated with reproductive physiology. Thus, *pou1f1* is a candidate gene for evolutionary developmental changes that are independent of reproductive maturation (neoteny), as is seen in the example of salamander paedomorphosis.

In contrast to *met3*, identifying candidates for *met1 and met2* has proven more difficult. For example, the 2 cm interval defining the location of *met1* on LG2 marks a unique synteny disruption in *Ambystoma* and perhaps other salamanders (Voss et al., 2011). This is a bit surprising and unlucky because the *Ambystoma* genome has undergone relatively few chromosomal rearrangements relative to other vertebrates and especially amniotes (Smith & Voss, 2006). Genes (e.g., *rai1*, *shmt1*, *drg2*, *med9*) from the Smith–Magenis disease syndrome region in the human genome flank one side of *met1*, while several genes associated with neural development and function (e.g., *setd2*, *ngfr*, *ccm2*) flank the other side. In no other vertebrate genome are these groups of flanking markers observed in synteny. Recently, we used microarray analysis to identify genes that are expressed differently during larval development as a function of *met1* genotype (Robert Page and Randal Voss, unpublished data). We reasoned that this combined genetic-genomic

approach would identify differentially expressed genes that map to the position of *met1*, potentially identifying candidate genes. As predicted, the majority of differentially expressed genes from LG2 mapped within 10 cm of *met1*, and six of the 14 genes that located within 2 cm of *met1* were differentially expressed as a function of genotype during larval development and/or metamorphosis. At this point, it is not clear if the *met1* genomic region is more enriched for differentially expressed genes than other regions of the *Ambystoma* genome, and perhaps genes that are TH responsive and associated with brain development. Regardless, it is clear from microarray studies that many genes are differentially expressed between paedomorphic *A. mexicanum* and metamorphic *A. t. tigrinum*, and *met1* locates to a uniquely structured genomic region with several candidate genes for paedomorphosis.

10. SYNTHESIS: EVOLUTION OF SALAMANDER PAEDOMORPHOSIS

Many times during salamander evolution, mechanisms that regulate TH induction of metamorphosis were altered, resulting in paedomorphic salamanders that retain larval traits into the adult, breeding phase. Over the past 100 years, researchers have treated paedomorphic salamanders with TH to investigate the physiological and evolutionary basis of this unique salamander trait. Studies show that tissues within some species are entirely resistant to TH, while others show partial or complete metamorphosis. These varied responses are not explained by phylogeny or morphology. Salamanders are a morphologically conservative group and paedomorphosis has evolved independently during salamander evolution. It is not likely that investigations of convergent morphological differences among deeply divergent paedomorphic species will yield a synthetic model for the origin of salamander paedomorphosis. Instead, an understanding of the origins of paedomorphosis requires knowledge of physiological and genetic mechanism, environmental context, and life history traits associated with fitness (Fig. 8.5). In the case of ambystomatid salamanders, paedomorph expression is associated with TH-responsive loci that pleiotropically affect metamorphic timing and adult fitness traits. According to our model, paedomorphosis and metamorphosis are mechanistically connected by loci that segregate allelic variation for responsiveness to T4. Phenotypes arising from hybrid ambystomatid crosses clearly show that it is possible to artificially phenocopy the complete spectrum of metamorphic timing variation observed among species in nature. Given variation in habitat stability, selection should favor

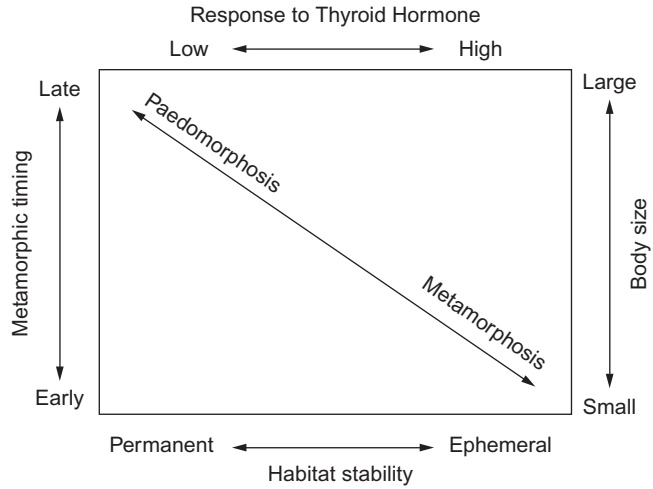

Figure 8.5 Model showing relationships among salamander life history variation, physiology, and habitat stability. Paedomorphosis is associated with low thyroid hormone responsiveness, permanent aquatic habitats, delayed metamorphic timing, and large body size. Metamorphosis is associated with contrary values for these variables.

low TH response alleles in biphasic populations when habitats allow for extensions of the larval period, as these alleles will delay metamorphic timing and increase adult body size. Under directional selection for mutations that increase the length of the larval period, central mechanisms of TH-release become progressively desensitized to intrinsic and extrinsic signals that provide reliable cues for initiating metamorphosis within the context of a biphasic life history. We hypothesize that central mechanisms are desensitized because TH-response loci become fixed for alleles that are less responsive to positive TH feedback. As genetic background changes, the likelihood of paedomorph expression increases. In support of this model, studies of facultative paedomorphic populations (*A. talpoideum*) have demonstrated the efficacy of selecting for higher paedomorph expression in relatively few generations (Semlitsch & Wilbur, 1989). If directional selection occurs over many generations and within the context of large and stable aquatic habitats, as has happened independently in the case of Tiger salamanders species in Mexico (Shaffer, 1984; Shaffer & McKnight, 1996; Shaffer & Voss, 1996), further changes in genetic background are predicted to yield individuals that are no longer capable of initiating metamorphosis in nature.

Many studies have shown that environmental factors, and especially low temperature, are often associated with paedomorphic populations (Eagleson & McKeown, 1980; Snyder, 1956; Sprules, 1974). It is possible that some examples

of paedomorphosis may be pathological, reflecting extreme physiological conditions that may be exacerbated by inbreeding depression within small, isolated populations. However, Matsuda (1982; 1987) suggested that low temperature is pivotal in the adaptive evolution of paedomorphic salamanders; low temperature initially causes the expression of paedomorphosis in a population, and then through genetic assimilation (Waddington, 1953) the paedomorphic phenotype becomes fixed. The model that we proposed above is consistent with genetic assimilation because we also assume directional selection of TH-response alleles that increase an individual's likelihood of expressing a paedomorphic phenotype. The selection process and allelic effects depend upon environmental context.

The experimental approaches and evolutionary model outlined above provide a possible framework from which to initiate studies of the genetic basis of metamorphic timing in natural amphibian populations. At this point, it is not clear how many TH-response genes contribute to adaptive differences in metamorphic timing within natural populations and among species, or whether the same loci and physiological mechanisms are implicated in independent examples of paedomorph evolution. Also, much remains to be learned about facultative paedomorphosis at a mechanistic level. For example, does facultative expression of paedomorphosis mechanistically present an intermediated condition between metamorphosis and paedomorphosis, and how is it regulated? It is exciting to think that these and other questions can now be resolved using genetic and genomic methods that are applicable to nongenetic model organisms. The future looks bright for investigating evolutionary, developmental, and physiological aspects of salamander life history and life cycle evolution.

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