EVOLUTION & DEVELOPMENT 7:5, 458–467 (2005)

Variation in thyroid hormone action and tissue content underlies species differences in the timing of metamorphosis in desert frogs

Daniel R. Buchholz* and Tyrone B. Hayes

Laboratory for Integrative Studies in Amphibian Biology, Museum of Vertebrate Zoology, Department of Integrative Biology, Group in Endocrinology, University of California, Berkeley, CA 94720-3140, USA

*Author for correspondence (email: buchhold@mail.nih.gov)

SUMMARY Hormonal control of post-embryonic morphogenesis is well established, but it is not clear how differences in developmental endocrinology between species may underlie animal diversity. We studied this issue by comparing metamorphic thyroid hormone (TH) physiology and gonad development across spadefoot toad species divergent in metamorphic rate. Tissue TH content, in vitro tail tip sensitivity to TH, and rates of TH-induced tail tip shrinkage correlated with species differences in larval period duration. Gonad differentiation occurred before metamorphosis in species with long larval periods and after metamorphosis in the species with short larval periods. These differences in TH physiology and gonad development, informed by phylogeny and ecology of spadefoot metamorphosis, provide evidence that selection for the short larval periods in spadefoot toads acted via TH physiology and led to dramatic heterochronic shifts in metamorphic climax relative to gonad development.

INTRODUCTION

A major goal of evolutionary developmental biology is to understand the developmental and molecular mechanisms underlying animal diversity (Hall 2003). Because hormones affect much of post-embryonic morphogenesis (Tata 1996; Yen 2001), changes in developmental endocrinology may be a major driving force in phenotypic diversification (Ketterson and Nolan 1999). Previous studies showed that character trait variation induced by hormonal manipulation in crickets (Zera et al. 1998), birds (Ketterson and Nolan 1999), lizards (Hews and Moore 1995), and voles (Oksanen et al. 2002) revealed the potential for hormone-related, microevolutionary effects on fitness in a single species. Other studies hypothesized a correlation between hormonal change and major evolutionary events that occurred in distantly related taxa: (1) changes in timing of juvenile hormone secretion may be associated with evolution of metamorphosis in insects (Truman and Riddiford 1999) and (2) changes in tissue sensitivity to thyroid hormone (TH) may underlie the large diversity in salamander cranial morphology (Rose 1996). However, endocrine differences as the basis for adaptive phenotypic evolution between closely related species has been difficult to identify.

Spadefoot toads, an ancient group of fossorial anurans, are comprised of two New World genera (the sister taxa *Scaphiopus* and *Spea*) and one Old World genus (*Pelobates*) (Cannatella 1985; Maglia 1998; Garcia-Paris et al. 2003). *Scaphiopus couchii* breeds in the most ephemeral desert pools among the spadefoot toads (Bragg 1945; Kuzmin 1999) and has the shortest larval period known among frogs (Newman 1989). The longest larval period recorded in nature for this species is 40 days (Wright and Wright 1949). Larval periods are 30% longer in the other genus of New World spadefoot toads, *Spea* (Buchholz and Hayes 2002), that breeds in longer lasting desert pools compared with pools used by *Sc. couchii* (Morey and Reznick 2004). Old World spadefoot toads, *Pelobates*, have larval periods twice as long as *Sc. couchii* under the same laboratory conditions (Buchholz and Hayes 2002), but in nature, the larval period of *Pelobates* is minimally several months in long-lasting vernal pools (Ugurtas 1995; Kuzmin 1999). Within every set of laboratory rearing conditions, *Sc. couchii* had a shorter larval period compared with *Sp. multiplicata* and *Pelobates syriacus* (Buchholz and Hayes 2000, 2002). For example, at 28°C with one tadpole per tank fed ground rabbit chow ad libitum, the larval period of *Sc. couchii* was 12 days, compared with 16 days for *Sp. multiplicata*, and 31 days for *Pb. syriacus*.

Such rapid metamorphosis in desert-dwelling *Scaphiopus* is an adaptation for desert survival (Bragg 1945; Gould and Vrba 1982; Green 1986). The laboratory rearing experiments by us and others revealed a genetic basis for rapid metamorphosis in *Sc. couchii* (Newman 1988; Buchholz and Hayes 2002; Morey and Reznick 2004). Also, the evolutionary appearance of short larval periods comes at the point of divergence between Old World (*Pelobates*) and New World (*Scaphiopus* and *Spea*) spadefoot taxa (Cannatella 1985;

Maglia 1998; Garcia-Paris et al. 2003). Furthermore, paleontological evidence provides a correlation between spadefoot divergence and the timing of North American aridification (Bragg 1961; Kluge 1966).

Frog metamorphosis is totally dependent upon TH (Dodd and Dodd 1976), and many features of TH physiology can be hypothesized to influence rate of metamorphosis and larval period duration. These hormonal influences can occur at the level of independently programmed and regulated tissue-specific ("peripheral") responses to TH and/or at the level of the hypothalamus–pituitary–thyroid gland axis, which controls tissue transformation "centrally" (Denver 1996; Shi et al. 1996). Whereas several studies have shown two species may differ in larval period duration when reared under laboratory conditions (Rafinska 1991; Gollmann and Gollmann 1993; Ptacek 1996), no previous study has examined potential endocrine basis for this difference or put such endocrine comparisons in a phylogenetic context. Here, we tested the hypothesis that differences in TH physiology, specifically tissue TH content and in vitro tissue sensitivity and responsivity to TH, correlate with differences in larval period duration between spadefoot toad species. In our previous study, we reared all spadefoot species but one in identical laboratory conditions and showed that species within a genus have similar larval periods (Buchholz and Hayes 2002). Therefore, we chose one species per genus for our endocrine experiments. In addition, TH has dramatic developmental effects on all organs of the body except the gonads (Ogielska and Kotusz 2004). Thus, we looked for changes in relative developmental timing in gonad differentiation consistent with evolutionary changes in TH physiology across spadefoot species with different larval period durations.

MATERIALS AND METHODS

Animal care and treatment

Adult *Sc. couchii* and *Sp. multiplicata* were collected under Arizona Scientific Collecting Permit #SP665546. *Pb. syriacus* adults were a kind gift from Dr. I. Ugurtas. Induction of breeding to obtain tadpoles was described previously (Buchholz and Hayes 2002). Two to four clutches of eggs per species were reared independently in 180 l of carbon-filtered tap water changed every other week at 24°C, and fed ground rabbit chow daily. At the indicated Gosner stages (Gosner 1960), 300 tadpoles were randomly selected from a pool. Adult care, tadpole rearing, and euthanasia were carried out in accordance with the Animal Use Protocol (R209-0498BR) issued to TBH and approved by the Office of Laboratory Animal Care and the Animal Care and Use Committee at UC Berkeley.

TH radioimmunoassay

At the indicated stages, tadpoles were immediately frozen or organs were dissected then frozen. TH extractions and T4 and T3 radioimmunoassays were carried out as described by Denver (1993), Hayes and Wu (1995). No samples were pooled. Mean recovery across taxa, tissue, and stage was 78% (range 53–95%) for T4 and 77% (range 48–97%) for T3. All assays were performed in duplicate. The range of detection was 2–600 pg/tube for both T4 and T3. Extraction of buffer alone gave potency estimates of zero or undetectable, and in each taxon, approximately 100% of cold T4 and T3 (with recoveries taken into account) was recovered when added to the homogenates. Potency estimates (pg/g) for the samples were calculated using a multiplier based on quality control samples to account for interassay variation and fell within two standard deviations of the ED50. Each radioimmunoassay run could accommodate 12 samples. The tissue samples for each run, namely tail and liver from each taxon at two developmental stages, were chosen so that interassay variation (3.8% for T4 and 5.0% for T3 ($n = 27$)) affected comparisons among species less than comparisons among stages. The samples (24 out of 641) that did not fall within the linear portion of the standard curve or that did not have slopes parallel with the standard curve were not included in the analyses. The ranges of $\%B/B_o$ within organ and hormone were overlapping across taxa. The sample size was four to 10 tails or livers per species at each stage, and significant differences between taxa within a developmental stage were determined by ANOVA followed by Scheffe's post hoc tests at a significance level of $\alpha = 0.05$ (Statview, Abacus Concepts).

Tail tip assay

The tail tip assay was based on methods described previously (Shaffer 1963; Derby 1975). When tadpoles achieved Gosner stage 28, 33, and 37, an overnight incubation in a 0.05% sulfadiazine (ICN, Costa Mesa, CA, USA) solution was used to help eliminate microorganisms. The next day, equally sized tail tips were cut 2–4 mm from the end of the tail and placed in 1.0 ml of 62% Leibovitz's-15 medium (L-15, Fisher, Pittsburg, PA, USA) with 10 × antibiotic–antimycotic solution (1000 U/ml penicillin G, 2.5 µg/ml amphotericin B, 1000 µg/ml streptomycin; Mediatech, VA, USA). After 2–3 h, the tail tips were transferred to 62% L-15 with 1 × antibiotic–antimycotic solution and incubated overnight at 28°C. After this incubation, tail tips were transferred to triiodothyronine (T3) treatments (0, 0.5, 1, 2, 4, or 8 nM T3) and remained in the treatments with no changes for 2 weeks. Tail tips at stage 28 from this clutch of *Sc. couchii* were lost because of contamination. Tail tip shrinkage was monitored daily for 14 days using a digital camera connected to a dissection microscope. Four tail tips were used for each species, stage, and T3 concentration. We repeated this entire experiment with a second clutch of tadpoles from each taxon using 0, 1, 2, 4, 8, or 150 nM T3, and also 0.5 nM T3 for *Sc. couchii* at stage 28. A third clutch was used from *Sp. multiplicata* at stage 37 using 0, 1, 2, 4, 8, or 150 nM T3. We considered tail tips to be sensitive to a concentration of T3 when three or four tail tips from a treatment group (species, stage, T3 concentration) shrank to less than 25% their original size. In the majority of treatments (90 of 104), either no tail tips shrank or three to four tail tips shrank. In only 14 out of 104 treatments, only one or two tail tips shrank to less than 25%.

We quantified the response of tail tips sensitive to T3 by determining the day on which shrinkage to 25% occurred. We found that three of the 40 treatment groups with repeat clutches differed

in sensitivity between clutches, where one clutch had less than half the tail tips shrink to less than 25% and the other clutch had more than half. These three cases occurred in *Pb. syriacus*: stage 28 at 4 and 8 nM T3 and stage 37 at 2 nM T3. Rather than combining these clutches, we show the data for the clutches with sensitive tail tips. Otherwise, we combined clutches for analysis. The sample size for response to T3 was four to 12 tail tips per species, stage, and T3 concentration. We determined significant shrinkage response (day of shrinkage to $<25\%$) between species within hormone treatment and stage using ANOVA followed by Scheffe's post hoc tests at a significance level of $\alpha = 0.05$ (Statview, Abacus Concepts). ANOVA and Scheffe's post hoc tests were also performed to identify significant differences between hormone treatments within *Sc. couchii*. As a positive control to show that tails of all taxa were responsive in this assay, tail tips from each species and each stage shrank within 2–4 days at 150 nM T3 (data not shown).

Gonad histology

At the indicated stages, tadpoles were fixed in neutral-buffered formalin and the kidney/gonad complexes were embedded in paraffin. The entire gonad length of 21–23 individuals per taxon (one to six gonads per indicated stage) were sectioned and stained with hematoxylin and eosin. Gonad differentiation occurs along an anterior–posterior gradient, so we noted the most advanced stage of differentiation within each gonad, which is found in the most anterior part of the gonad. Gonads were identified as testes (reduced cortex), ovaries (ovarian cavity), or undifferentiated gonads (well developed cortex and medulla) (Hayes 1998). Comparisons between taxa were qualitative to determine whether sexual differentiation occurred before or after tail resorption.

RESULTS

Couch's spadefoot toad *Sc. couchii* has the shortest larval period known among frogs and metamorphoses faster than the New Mexican spadefoot *Sp. multiplicata* and the Eastern European spadefoot toad *Pelobates syriacus* when reared under identical laboratory conditions (Fig. 1A and data not shown). First, we confirmed that an increase in the active form of TH, triiodothyronine (T3), correlated with metamorphosis in *Sc. couchii* (Fig. 1B), as in other spadefoot toads and all anuran taxa studied to date (Denver 1993, 1998). Whole body T3 contents of *Sc. couchii* revealed the expected increase during the larval period, as seen for whole body measurements in *Bufo* (Weber et al. 1994) and plasma measurements in *Rana* and *Xenopus* (Leloup and Buscaglia 1977; Regard et al. 1978; Mondou and Kaltenbach 1979). In addition, as in other taxa (Dodd and Dodd 1976; White and Nicoll 1981), metamorphosis of *Sc. couchii* and *Sp. multiplicata* was inhibited by thiourea (data not shown), a chemical that blocks TH synthesis, further indicating that TH is required for metamorphosis in spadefoot toads.

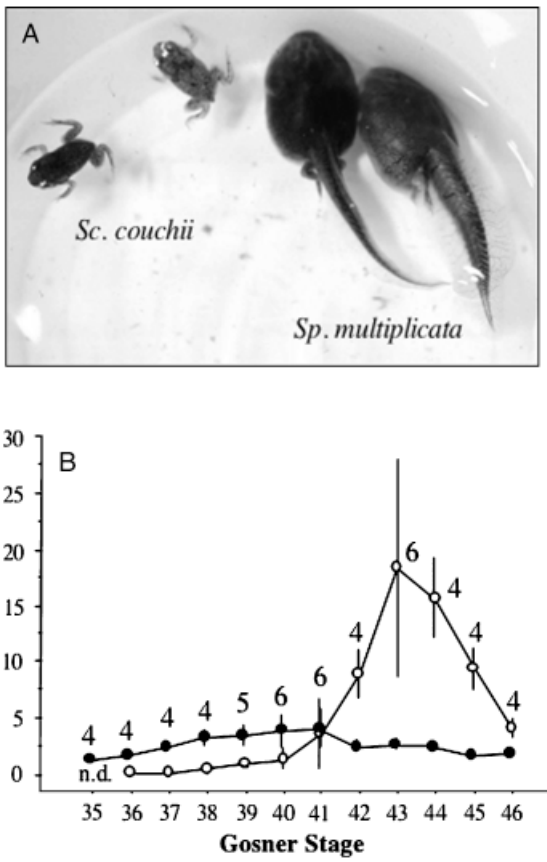

Fig. 1. Rapid metamorphosis and thyroid hormone (TH) content in Couch's spadefoot toad, *Scaphiopus couchii*. (A) Individuals of *Sc. couchii* (day of tail resorption) and *Spea multiplicata* (prometamorphic tadpoles) exhibit dramatically different durations of the larval period. Tadpoles of both species were reared under identical laboratory conditions for 12 days (32°C, 10 tadpoles in 4 l, and fed ground rabbit chow ad libitum). Snout-vent length of *Sc. couchii* is 12 mm. Tadpoles of *Pelobates syriacus* reared under the same conditions had not begun metamorphosis (not shown). (B) Whole body triiodothyronine (T3) content in *Sc. couchii* showed the expected peak at climax of metamorphosis, stage 43, lacking any correlation with mass. Data points represent mean and standard deviation of the sample sizes noted at each stage. n.d., not detected.

We hypothesized an endocrine basis for the short larval periods in *Scaphiopus*. Classical endocrine experiments showed that the rate of TH-induced morphological change correlates with the amount of exogenous hormone administered to premetamorphic tadpoles (Kollros 1961). Therefore, different levels of endogenous TH across taxa would be expected to correlate with larval period differences. To test this hypothesis, we took tail and liver tissues from *Sc. couchii*, *Sp. multiplicata*, and *Pb. syriacus* throughout metamorphosis and measured by radioimmunoassay the precursor hormone thyroxine, T4, and triiodothyronine, T3. T4 is the major product secreted by the thyroid gland and T3 is metabolized from T4 by peripheral tissues. Some tadpoles from each species were

followed through metamorphosis to confirm that, under the rearing conditions used for the endocrine comparisons, differences in larval period among species were observed as seen before (Buchholz and Hayes 2002). The time when approximately half the cohort reached tail resorption was 23 days for *Sc. couchii*, 41 days for *Sp. multiplicata*, and 59 days for *Pb. syriacus*.

Comparisons of tail and liver T4 and T3 contents across spadefoot taxa were used in lieu of plasma hormone concentrations, because tadpoles of *Sc. couchii* are too small to obtain plasma samples. Also, whole body analysis was not used because gut volumes vary nonisometrically across species and developmental stage and thus would yield hormone contents inappropriate for comparisons made here (Denver 1993; Buchholz and Hayes 2002). Similar to previous hormone profiles, tail and liver tissue contents from each taxon showed a peak in T4 and T3 at metamorphic climax, stage 42–43 (Fig. 2). Also, tissue amounts of T4 were 2- to 4-fold higher than amounts of T3 in both the tail and liver, similar to studies using whole bodies or plasma (Leloup and Buscaglia 1977; Regard et al. 1978; Mondou and Kaltenbach 1979; Weber et al. 1994).

In the tail, *Sc. couchii* had 1.7- to 4.9-fold higher T4 contents for all stages and was significantly higher in four of the nine stages measured during metamorphosis compared with *Pb. syriacus* (Fig. 2A). For T3, the active form of TH, *Sc. couchii* had 1.6- to 5.1-fold higher contents for all stages and was significantly higher in four of the six stages measured compared with *Pb. syriacus* (Fig. 2B). The higher T4 and T3 tail contents in *Sc. couchii* correlated with its shorter larval period length compared with *Pb. syriacus* (Buchholz and Hayes 2002). In contrast to the tail, there was no consistent difference in tissue content of T3 in the liver between *Sc. couchii* and *Pb. syriacus* during metamorphosis (Fig. 2D).

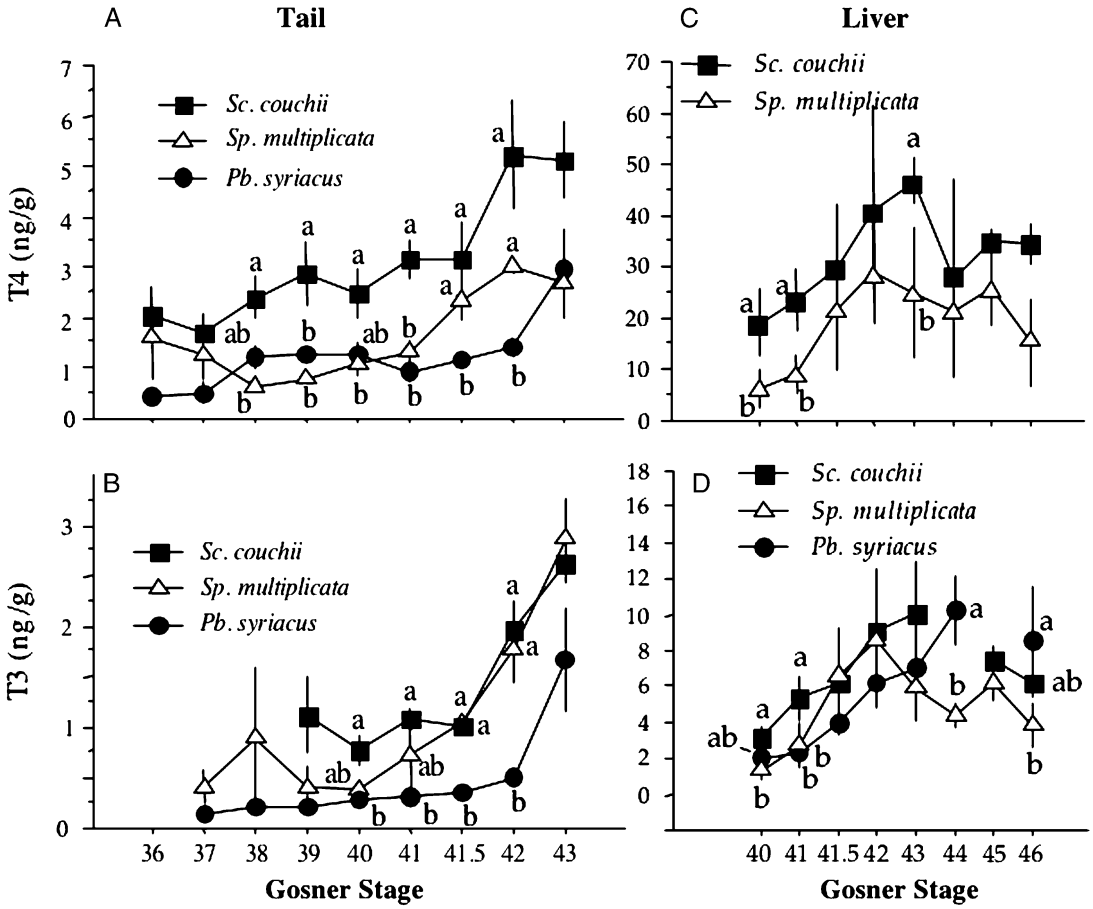

Fig. 2. Tail and liver tissue content of thyroxine (T4) and triiodothyronine (T3) during metamorphosis in spadefoot toads. Tail tissue content of T4 (A) and T3 (B) increased with developmental stage and was highest in *Scaphiopus couchii* by 2- to 5-fold compared with *Pelobates syriacus*. Liver tissue content of T4 (C) and T3 (D) during metamorphosis in spadefoot toads also increased with developmental stage. Data for T4 liver content of *Pb. syriacus* did not fall in the linear portion of the curve. Unlike the tail data, the liver data do not show consistent differences among taxa. Each data point represents the mean and standard deviation from four to ten tails or livers. Letters indicate significance groups among taxa within stage based on Scheffe's post hoc tests at a significance level of $\alpha = 0.05$.

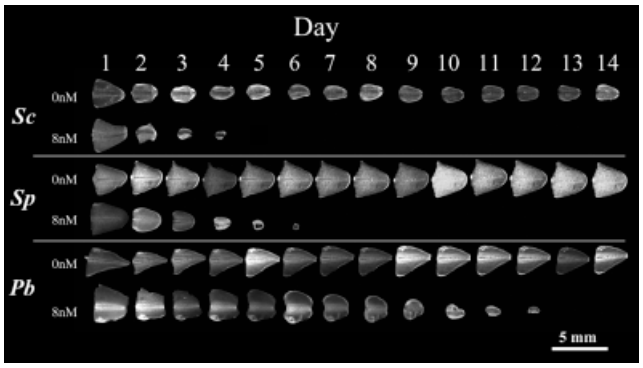

Fig. 3. Tail tip shrinkage in vitro in response to TH across taxa. Tail tips from *Saphiopus couchii*, *Spea multiplicata*, and *Pelobates syriacus* at Gosner stage 33 were treated with triiodothyronine (T3) at 0 and 8 nM in vitro on day 1 and tail shrinkage was monitored daily for 14 days using a digital camera connected to a dissection microscope. The representative tail tips shown here illustrate that tail tips from *Sc. couchii* shrank faster than *Pb. syriacus* in the presence of T3 and that the tail tips of *Sp. multiplicata* shrank at an intermediate rate.

Thus, the differences between these two taxa in TH contents were organ-dependent, that is, *Sc. couchii* had higher amounts of TH in the tail, not in the liver.

Hormone content in livers and tails of *Sp. multiplicata* were, in general, not significantly different from those of *Sc. couchii* or *Pb. syriacus* (Fig. 2). In *Sp. multiplicata* compared with *Pb. syriacus,* significantly higher tail T4 and T3 contents were limited to stage 41.5 and 42 at the climax of metamorphosis. Compared with *Sc. couchii*, *Sp. multiplicata* had significantly lower T4 levels during some prometamorphic stages (38–41), but there was no significant difference in the active hormone T3 throughout metamorphosis.

Because *Sc. couchii* and *Sp. multiplicata* differ significantly in larval period yet lack significant differences in TH content, we compared a second feature of TH physiology that may differ across spadefoot species and may contribute to differences in larval period, namely, tissue sensitivity and responsivity to T3. Within an individual, the sequence of metamorphic transformations from limbs to intestine to tail indicates that different tissues respond to the same plasma concentration of TH differently. We hypothesized that tissues in species with shorter larval periods will have higher tissue sensitivity and/or responsivity to T3 compared with species with longer larval periods. We compared tail tissue sensitivity and analyzed tail tip shrinkage in response to T3 using an in vitro tail tip assay with seven concentrations of T3 at three developmental stages in *Sc. couchii*, *Sp. multiplicata*, and *Pb. syriacus* (representative tail tips shown in Fig. 3). *Sc. couchii* was sensitive to as low as 1 nM T3 across all three stages tested, compared with 2–8 nM T3 for the other taxa, depending on species and stage (Table 1). In addition, at 4 and 8 nM T3, the rate of tail shrinkage in *Sc. couchii* averaged about 2-fold higher than the shrinkage rates in *Sp. multiplicata* and *Pb. syriacus*. These results, consistent across all three stages and experiments, indicated that *Sc. couchii* had higher sensitivity and faster response kinetics than the other taxa, correlating with its shorter larval period.

The differences in tail tip shrinkage between *Sp. multiplicata* and *Pb. syriacus* were less dramatic. Sensitivity to 2 nM T3 did not occur in *Sp. multiplicata* but did occur at stage 37 in *Pb. syriacus* in only one of two clutches. Also, the sensitivity to 4 nM T3 was not consistent across clutches in *Pb. syriacus* at stage 28 or across the three stages in *Sp. multiplicata* and *Pb. syriacus*. Thus, it seems 2–4 nM represents the limit of sensitivity for these two species in this assay

Table 1. Sensitivity and responsivity of tail tips to TH *in vitro* at stage 28, 33, and 37 in *Scaphiopus couchii*, *Spea multiplicata*, and *Pelobates syriacus*

	Stage 28			Stage 33			Stage 37		
T3 (nM)	*Sc.*	*Sp.*	*Pb.*	*Sc.*	*Sp.*	*Pb.*	*Sc.*	*Sp.*	*Pb.*
0	—	—	—	—	—	—	—	—	—
0.5	—	—	—	—	—	—	—	—	—
1.0	8 ± 2	—	—	7 ± 2	—	—	10 ± 3	—	—
2.0	7 ± 2	—	—	5 ± 1	—	—	6 ± 1	—	11 ± 1
4.0	5.3 ± 0.5	—	12.7 ± 0.6	4.6 ± 0.8	10 ± 3	—	4.8 ± 0.7	8 ± 2	9 ± 2
8.0	3.3 ± 0.5	6.1 ± 0.9	9 ± 1	3.4 ± 0.5	5 ± 1	8 ± 1	3.4 ± 0.5	6 ± 2	8 ± 2

Triiodothyronine (T3) at the indicated concentrations was added to the tail tip cultures on day 1, and tail tip size was measured daily for 14 days. Long dashes represent T3 concentrations at which tail tips were not sensitive, that is, did not shrink to 25% original size of the tail tip. Numbers in table signify the day (mean ± SD) on which shrinkage occurred to less than 25% the original size of the tail tip. Sample size was four to 12 tail tips per species, stage, and T3 concentration. Solid boxes around data points for *Sc. couchii* refer to significance groups across T3 concentrations, and dashed boxes indicate significance groups across species at 8 nM T3 (Scheffe's post hoc test, $P<0.05$). At each T3 concentration and stage, tail tips of *Sc. couchii* shrank significantly faster than the other taxa.

and do not provide consistent evidence for differences between them. At 8 nM T3, tail tips of both species were sensitive across all three stages, and response time of shrinkage to 25% original size was significantly different between the two species at stages 28 and 33, but not stage 37, thus lending weak support for an endocrine difference between *Sp. multiplicata* and *Pb. syriacus* correlating with their different larval periods.

Because endocrine physiology is characterized by multiple or pleiotropic effects throughout the organism (Ketterson and Nolan 1999), evolutionary changes in TH physiology may result in heterochronic shifts in developmental events with respect to each other. We examined developmental timing of gonad differentiation across spadefoot taxa to look for developmental patterns consistent with endocrine-based evolution of rapid metamorphosis. Our histological analysis of 21–23 spadefoot toad gonads per species (one to six gonads per indicated stage) during the larval period at or before tail resorption showed that both *Spea* and *Pelobates* had differentiated gonads before tail resorption (Fig. 4, A–D). For *Pb. syriacus*, we examined 21 gonads of pre- and prometamorphic tadpoles (stages 31–39), and 17 were already differentiated. The four undifferentiated gonads were observed in early premetamorphosis (stages 31–33) (Fig. 5A). Similarly, we examined 17 pre- and prometamorphic gonads (stages 32–39) for *Sp. multiplicata*, and only five were not already differentiated (stages 32–37) (Fig. 5B). All six gonads of *Sp. multiplicata* examined from climax of metamorphosis (stages 40–46) were already differentiated. In contrast, we examined 23 gonads of *Sc. couchii*, all from climax of metamorphosis, and none were differentiated, even though 12 of these gonads were from the last two stages of metamorphosis (stage 46 is completion of tail resorption) (Fig. 5C). Thus, the gonads are not differentiated before metamorphosis in *Sc. couchii*, whereas gonads differentiate during the larval period in *Sp. multiplicata* and *Pb. syriacus*. Gonad differentiation after tail resorption was not followed in *Sc. couchii*, but gonads sectioned after two months of post-metamorphic growth showed an advanced stage of differentiation (Fig. 4F).

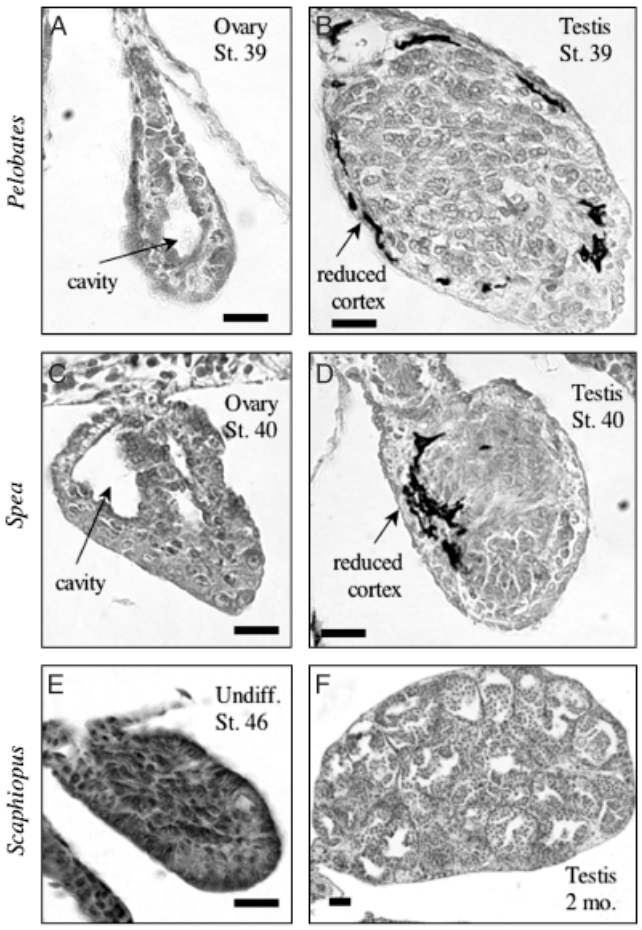

Fig. 4. Gonad differentiation in spadefoot toads. (A) Ovary and (B) testis of *Pelobates syriacus* differentiated before metamorphic climax, stage 39. (C) Ovary and (D) testis of *Spea multiplicata* differentiated before metamorphic climax, stage 40. Gonads of *Scaphiopus couchii* remained undifferentiated at tail resorption, stage 46 (E). Note the ovarian cavity in (A) and (C) and the reduced cortex in (B) and (D), whereas the cortex and medulla are both intact in gonads of *Sc. couchii* (E) (Hayes 1998). By two months post-metamorphosis, seminiferous tubules in gonads of *Sc. couchii* (F) indicate development to a stage well beyond that shown for *Pb. syriacus* and *Sp. multiplicata*. Scale bar, 30 μm.

DISCUSSION

Animal morphology is spectacular in its diversity, and developmental endocrinology underlies the generation of animal form (Tata 1996). However, little is known about how changes in hormone physiology affect evolutionary diversity. The role of hormones in development is complex because of the pleiotropic nature of hormone action and epistatic effects of hormones on phenotypes (Ketterson and Nolan 1999), so that the ways endocrine systems can evolve and the morphological consequences of endocrine evolution are not clear. Using spadefoot toad metamorphosis where close relatives exhibit a high degree of larval period diversity (Buchholz and Hayes 2002), we showed first that whole body TH contents increased across development in *Sc. couchii* indicating its metamorphosis is controlled by TH as in *Spea* (Denver 1993, 1998) and all other frog species studied to date (Shi 1999). Then, we compared two features of TH physiology that may contribute to larval period differences between species. Further, we found differences in timing of gonad development relative to metamorphic climax across species divergent in larval period duration consistent with evolutionary shortening of larval period via changes in TH physiology. We incorporated phylogeny, ecology, development, and endocrinology into our understanding of the evolution and endocrinology of accelerated metamorphosis in spadefoot toads (Fig. 6).

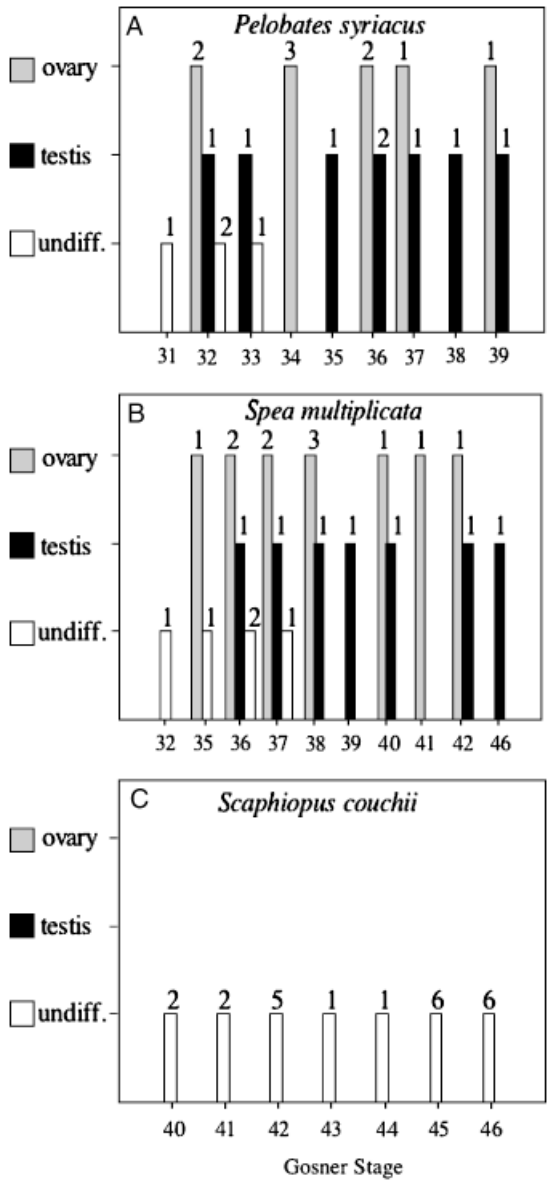

Fig. 5. Timing of gonad differentiation in spadefoot toads. At the indicated stages and sample sizes, gonads of (A) *Pelobates syriacus*, (B) *Spea multiplicata*, (C) *Scaphiopus couchii*, were identified as testes (cortex regression), ovaries (ovarian follicles), or undifferentiated gonads (intact cortex and medulla) (Hayes 1998). Note that gonad differentiation occurs well before tail resorption in *Pb. syriacus* and *Sp. multiplicata* but not in *Sc. couchii*.

TH is critical for frog metamorphosis (Dodd and Dodd 1976), and rate of metamorphosis correlates with TH concentration added to the rearing water (Kollros 1961). Also, studies on tadpoles reared under conditions that increase metamorphic rate have found increased TH content correlating with shorter larval periods (less than 2-fold at the same developmental stage) in *Sp. hammondii* (Denver 1998) and *B. calamita* (Gomez-Mestre et al. 2004). Ours is the first to study the hypothesis that differences in endogenous tissue TH content correlate with different larval period durations between species reared under identical laboratory conditions. Because completion of tail resorption during climax of metamorphosis is a major determinant of metamorphic progress and defines the end of metamorphosis (Gosner 1960), species differences in tail TH content may contribute to larval period differences. Our radioimmunoassay data for the tail showed that *Sc. couchii*, with its shorter larval period, had significantly higher tail TH content, particularly of the active form T3 by 3-fold on average, across most of metamorphosis compared with *Pb. syriacus*. Analogous to classical endocrine experiments where rate of metamorphic transformation correlates with concentration of endogenous T3, higher tail contents of TH support our hypothesis and suggest that higher tail tissue content of TH is a contributing endocrine mechanism to the shorter larval period found in *Sc. couchii* compared with *Pb. syriacus*.

We also measured T3 content in the liver and expected similar data as in the tail. However, we found that, for the most part, there was little difference between spadefoot species across metamorphosis, a result contrasting with the data from tail hormone measurements. Our radioimmunoassay data showing differences in tail TH content across species may suggest differences should be consistent across all organs because centrally controlled plasma TH levels are the same throughout the animal. However, tissue TH content may not reflect plasma TH levels. For example, tissue-specific levels of TH transport, cytosolic TH-binding protein, and/or metabolism of TH (Shi et al. 1996) may alter the effective TH content in tissues with respect to plasma levels. Thus, future experiments are required to identify differences in tissue-specific control of TH content among species to explain the presence of species differences in tail TH content and the absence of differences in liver T3 content. For example, future molecular studies may reveal differences between species in levels of TH transporters and/or T4 monodeiodinase in the tail but not in the liver, correlating with measurements of tissue TH content across species.

Tissue content of TH is likely not the only feature of TH physiology underlying larval period differences between species. The larval period of *Sp. multiplicata* is longer than that of *Sc. couchii*, but the T3 content of *Sp. multiplicata* was not statistically different from *Sc. couchii* across development. Thus, we examined another feature of TH physiology that may underlie larval period differences, tissue sensitivity and responsivity to TH. Just as rates of metamorphosis differ across tissues within an individual, as evidenced by developmental asynchrony of different tissues during metamorphosis, we hypothesized that rates of metamorphic transformation in the same tissue across taxa would correlate with larval period duration. Using an in vitro tail tip assay, we found that *Sc. couchii* with the shortest larval period had tails with higher sensitivity to TH and shrank significantly faster to TH

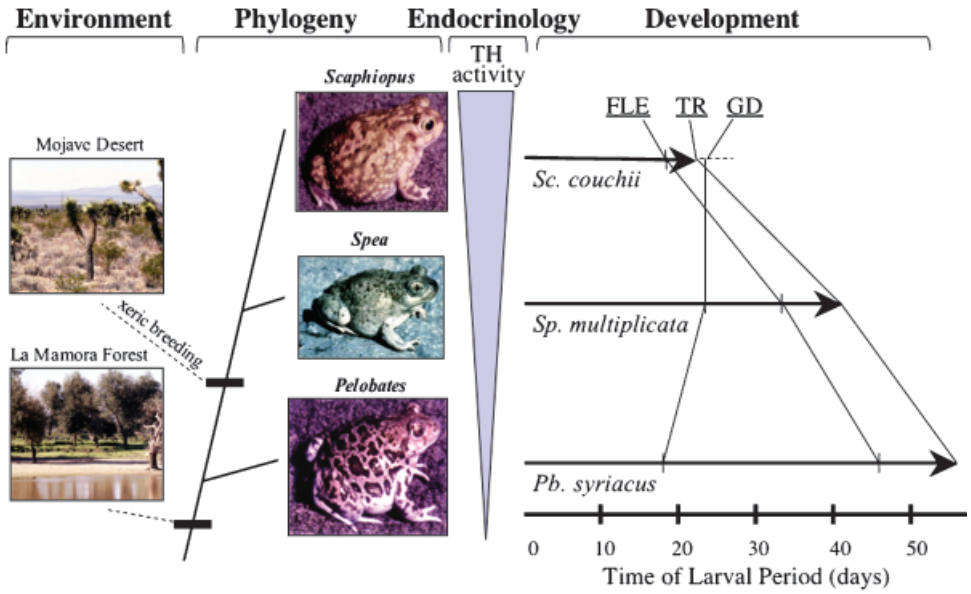

Fig. 6. Integration of endocrine data with ecology, phylogeny, and development. The New World spadefoot toads, *Scaphiopus couchii* and *Spea multiplicata*, breed during summer monsoons in arid environments, represented by the Mojave Desert in southeastern California. In contrast, Old World spadefoot toads, *Pelobates*, breed in long-lasting vernal pools in winter and spring, represented by a breeding pool of *Pelobates varaldii* from La Mamora Forest, Morocco. The cladogram shows the sister taxon relationship between *Scaphiopus* and *Spea*, with *Pelobates* basal, and indicates that the evolutionary origin of xeric breeding habits occurred within the spadefoot toads (Bragg 1961; Cannatella 1985; Buchholz and Hayes 2002). Our endocrine data are represented by a wedge of endocrine potency, where *Sc. couchii* has the most active thyroid physiology (i.e., higher tail TH content, higher tissue sensitivity and response to TH), *Sp. multiplicata* has an intermediate thyroid physiology, and *Pb. syriacus* has the least active thyroid physiology. The TH activity across taxa correlates with the duration of the larval period (Buchholz and Hayes 2002), shown by the time line to the right. The time line also depicts two measures of metamorphic rate (time to forelimb emergence (FLE) and tail resorption (TR)) juxtaposed with the timing of gonad differentiation (GD). Our model for the evolution of rapid metamorphosis in spadefoot toads is that selection for short larval periods to avoid desiccation in the ancestors of New World spadefoot toads, especially *Scaphiopus*, acted via changes in TH physiology and that this endocrine evolution resulted in accelerated development of all tissues except the gonads, which differentiated at their ancestral rate.

compared with *Sp. multiplicata* and *Pb. syriacus* across all three stages tested.

We suggest these differences in tail sensitivity/responsivity in *Sc. couchii* contribute to its shorter larval period because tail shrinkage is a major component in determining larval period duration. The different shrinkage rates among species imply peripheral mechanisms underlying sensitivity and responsivity of tail tips among species. Thus, similar to tail TH content differences among species, the tail tip experiments provide the framework for future molecular studies on tissue-specific molecular mechanisms for why species differ in tissue sensitivity/responsivity to TH.

Our endocrine data, in sum, pointed to differences in both tail TH content and tail sensitivity and responsivity to TH as likely endocrine mechanisms underlying the larval period differences between *Sc. couchii* and *Pb. syriacus*. However, only tail sensitivity/responsivity differences were suggested to contribute to differences in larval period between *Sc. couchii* and *Sp. multiplicata*. The few but significant differences in TH physiology between *Sp. multiplicata* and *Pb. syriacus* may impart biologically significant differences that underlie their different larval periods. Alternatively, additional developmental and physiological mechanisms may underlie the shorter larval period in *Sp. multiplicata* compared with *Pb. syriacus* (and longer larval period compared with *Sc. couchii*). For example, even though their tissue TH content is similar across developmental stages, *Sp. multiplicata* reached those stages earlier than *Pb. syriacus*, suggesting the developmental timing of maturation of the hypothalamus–pituitary–thyroid axis, which initiates metamorphic progression, may occur earlier in *Sp. multiplicata* than in *Pb. syriacus*. Also, effects of other hormones, such as prolactin and glucocorticoids, that inhibit or accelerate, respectively, the action of TH on metamorphosis (Kikuyama et al. 1993), could be additional species differences contributing their different larval periods.

The differences in TH physiology we identified that correlate with and likely contribute to larval period differences among spadefoot species are consistent with the idea that selection favoring rapid metamorphosis to avoid desiccation in *Sc. couchii* acted, at least in part, through TH physiology. Because *Spea*, *Pelobates*, and *Pelodytes*, the three nearest out-groups to *Scaphiopus*, have long larval periods relative to

Scaphiopus (Buchholz and Hayes 2002), the ancestors of *Scaphiopus* likely had long larval periods. Also, it is likely that the ancestors of *Sc. couchii* with long larval periods also had a TH physiology to match, because our endocrine study indicated a correlation of longer larval periods with lower tissue TH content and TH sensitivity/responsivity among spadefoot toads. Thus, we suggest that endocrine evolution in TH physiology occurred in the ancestors of *Scaphiopus* to achieve its short larval periods. Further support for this correlation between activity of TH physiology and larval period duration would require endocrine examination of other frog groups which contain multiple species with different larval periods.

To add support from a different line of evidence to our hormone data, which suggested that changes in TH physiology contributed to the evolution of shortened larval periods of *Sc. couchii*, we looked for possible developmental consequences consistent with evolutionary changes in TH physiology on development. We compared timing of gonad differentiation across spadefoot taxa because it is not affected by TH or stage of somatic development (Allen 1918; Swingle 1918; Kawahara et al. 1991; Robertson and Kelley 1992; Ogielska and Kotusz 2004; Rot-Nikcevic and Wassersug 2004). In those experiments, lack of TH or addition of exogenous TH did not affect the absolute age of the tadpole at which gonad differentiation occurred, even though there was a dramatic effect on the metamorphic stage at which this differentiation took place. Our histological study of spadefoot gonads showed dramatic differences in the stage at which the gonad differentiation occurs across species. Rather than identifying an exact stage or range of stages for gonad differentiation, our data show that the gonads of *Pb. syriacus* and *Sp. multiplicata* differentiated well before the end of metamorphosis. In contrast, differentiated gonads were not seen throughout the larval period of *Sc. couchii*.

We suggest that the endocrine evolution that produced the variation in TH physiology across spadefoot toads resulted in the shift in relative timing of metamorphosis and gonad differentiation among spadefoot toads. Our endocrine data, especially the in-vitro tail tip assay, imply that changes to attain the short larval periods in *Sc. couchii* occurred on a tissue-by-tissue basis. We suggest that an increase in tissue TH content and/or changes in mechanisms that increase tissue sensitivity/responsivity occurred in the ancestors of *Sc. couchii*. These changes caused a shortening of the larval period, and at the same time, caused TH-responsive tissues to "accelerate around" gonad differentiation. That is, the higher tissue response to higher TH levels led to more rapid transformation in TH-responsive tissues whereas gonad development remained to develop at the ancestral rate. We view the heterochronic shift in metamorphosis with respect to gonad development in *Sc. couchii* consistent with evolutionary changes in TH physiology, which allowed for accelerated metamorphosis. Further support for a role of endocrine evolution in larval period differences between species would come from a comparison of TH physiology and gonad development in other closely related taxa that differ in larval period duration.

As in sexual dichromatism in *Hyperolius* (Hayes 1997), melanocortin receptors in color variations in related lineages of reptiles and birds (Mundy et al. 2004; Rosenblum et al. 2004), and secondary sexual characteristics in fanged frogs (Emerson 2000), our study on how variation in TH physiology may underlie differences between species in larval period and the relative timing of gonad differentiation exemplifies the power of putting the endocrine control of development into a comparative and ecological/adaptive context to understand the role of hormones in the evolution of diversity.

Acknowledgments

Special thanks go to Dr. I. Ugurtas for invaluable help with *Pb. syriacus*. We also appreciate the careful critiques of previous drafts by R. Denver, Y.-B. Shi, M.-T. Nguyen, L. Fu, I. Gomez-Mestre, and anonymous reviewers. D. R. B. was an HHMI predoctoral fellow. This work was supported by NSF Grants IBN-9513362 and IBN-9508996 to T. B. H., by National Geographic Society Grant 6101-98 to T. B. H. and D. R. B., and by Museum of Vertebrate Zoology Louise Kellogg Grants-in-Aid, National Science Foundation Dissertation Improvement Grant, and Group in Endocrinology Howard Bern Award to D. R. B.

REFERENCES

Allen, B. M. 1918. The results of thyroid removal in the larvae of *Rana pipiens*. *J. Exp. Zool*. 24: 499–519.

Bragg, A. N. 1945. The spadefoot toads in Oklahoma with a summary of our knowledge of the group. *Am. Nat*. 79: 52–72.

Bragg, A. N. 1961. A theory of the origin of spade-footed toads deduced principally by a study of their habits. *Anim. Behav*. 9: 178–186.

Buchholz, D. R., and Hayes, T. B. 2000. Larval period comparison for the spadefoot toads *Scaphiopus couchii* and *Spea multiplicata* (Pelobatidae: Anura). *Herpetologica* 56: 455–468.

Buchholz, D. R., and Hayes, T. B. 2002. Evolutionary patterns of diversity in spadefoot toad metamorphosis (Anura: Pelobatidae). *Copeia* 2002: 180–189.

Cannatella, D. C. (1985). A phylogeny of primitive frogs (archaeobatrachians). Ph.D. Dissertation, University of Kansas, Lawrence, KS, USA.

Denver, R. J. 1993. Acceleration of anuran metamorphosis by corticotropin-releasing hormone-like peptides. *Gen. Comp. Endocrinol*. 91: 38–51.

Denver, R. J. 1996. Neuroendocrine control of amphibian metamorphosis. In L. I. Gilbert, J. R. Tata, and B. G. Atkinson (eds.). *Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells*. Academic Press, San Diego, CA, pp. 433–464.

Denver, R. J. 1998. Hormonal correlates of environmentally induced metamorphosis in the western spadefoot toad, *Scaphiopus hammondii*. *Gen. Comp. Endocrinol*. 110: 326–336.

Derby, A. 1975. An *in vitro* quantitative analysis of the response of tadpole tissue to thyroxine. *J. Exp. Zool*. 168: 147–156.

Dodd, M. H. I., and Dodd, J. M. 1976. The biology of metamorphosis. In B. Lofts (ed.). *Physiology of Amphibia*. Vol. 3. Academic Press, New York, NY, USA, pp. 467–599.

Emerson, S. B. 2000. Vertebrate secondary sexual characteristics—physiological mechanisms and evolutionary patterns. *Am. Nat*. 156: 84–91.

Garcia-Paris, M., Buchholz, D. R., and Parra-Olea, G. 2003. Phylogenetic relationships of the Pelobatoidea re-examined using mitochondrial DNA. *Mol. Phylogenet. Evol*. 28: 12–23.

Gollmann, B., and Gollmann, G. 1993. A laboratory experiment on interspecific competition between tadpoles of *Geocrinia victoriana* and *Pseudophryne semimarmorata* (Anura, Myobatrachinae). *Amphibia-Reptilia* 14: 349–356.

Gomez-Mestre, I., Tejedo, M., Ramayo, E., and Estepa, J. 2004. Developmental alterations and osmoregulatory physiology of a larval anuran under osmotic stress. *Physiol. Biochem. Zool.* 77: 267–274.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16: 183–190.

Gould, S. J., and Vrba, E. S. 1982. Exaptation: a missing term in the science of form. *Paleobiology* 8: 4–15.

Green, H. W. 1986. Diet and arboreality in the emerald monitor, *Varanus prasinus*, with comments on the study of adaptation. *Fieldiana Zool.* 31: 1–12.

Hall, B. K. 2003. Evo-Devo: evolutionary developmental mechanisms. *Int. J. Dev. Biol.* 47: 491–495.

Hayes, T. B. 1997. Hormonal mechanisms as potential constraints on evolution: examples from the Anura. *Am. Zool.* 37: 482–490.

Hayes, T. B. 1998. Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanism. *J. Exp. Zool.* 281: 373–399.

Hayes, T. B., and Wu, T. H. 1995. Interdependence of corticosterone and thyroid hormones in toad larvae (*Bufo boreas*). II. Regulation of corticosterone and thyroid hormones. *J. Exp. Zool.* 27: 103–111.

Hews, D. K., and Moore, M. C. 1995. Influence of androgens on differentiation of secondary sex characteristics in tree lizards, *Urosaurus ornatus*. *Gen. Comp. Endocrinol.* 97: 86–102.

Kawahara, A., Baker, B. S., and Tata, J. R. 1991. Developmental and regional expression of thyroid hormone receptor genes during *Xenopus* metamorphosis. *Development* 111: 933–943.

Ketterson, E. D., and Nolan, V. 1999. Adaptation, exaptation, and constraint: a hormonal perspective. *Am. Nat.* 154: S4–S25.

Kikuyama, S., Kawamura, K., Tanaka, S., and Yamamoto, K. 1993. Aspects of amphibian metamorphosis: hormonal control. *Int. Rev. Cytol.* 145: 105–148.

Kluge, A. G. 1966. A new pelobatine frog from the lower Miocene of South Dakota with a discussion of the evolution of the *Scaphiopus–Spea* complex. *Contrib. Sci., Los Angeles County Museum Nat. His.* 113: 1–26.

Kollros, J. J. 1961. Mechanisms of amphibian metamorphosis: hormones. *Am. Zool.* 1: 107–114.

Kuzmin, S. L. 1999. *The Amphibians of the Former Soviet Union, Pensoft Series Faunistica*. Vol. 12. Pensoft, Sofiya, Bulgaria.

Leloup, J., and Buscaglia, M. 1977. La triiodothyronine, hormone de la metamorphose des Amphibiens. *C. R. Acad. Sci. Paris, Ser. D* 284: 2261–2263.

Maglia, A. M. 1998. Phylogenetic relationships of extant pelobatoid frogs (Anura: Pelobatoidea): evidence from adult morphology. *Sci. Papers Nat. Hist. Mus. Univ. Kansas* 10: 1–19.

Mondou, P. M., and Kaltenbach, J. C. 1979. Thyroxine concentrations in blood serum and pericardial fluid of metamorphosing tadpoles and of adult frogs. *Gen. Comp. Endocrinol.* 39: 343–349.

Morey, S. R., and Reznick, D. N. 2004. The relationship between habitat permanence and larval development in California spadefoot toads: field and laboratory comparisons of developmental plasticity. *OIKOS* 104: 172–190.

Mundy, N. I., Badcock, N. S., Hart, T., Scribner, K., Janssen, K., and Nadeau, N. J. 2004. Conserved genetic basis of a quantitative plumage trait involved in mate choice. *Science* 303: 1870–1873.

Newman, R. A. 1988. Genetic variation for larval anuran (*Scaphiopus couchii*) development time in an uncertain environment. *Evolution* 42: 763–773.

Newman, R. A. 1989. Developmental plasticity of *Scaphiopus couchii* tadpoles in an unpredictable environment. *Ecology* 70: 1775–1787.

Ogielska, M., and Kotusz, A. 2004. Pattern and rate of ovary differentiation with reference to somatic development in anuran amphibians. *J. Morphol.* 259: 41–54.

Oksanen, T. A., Koskela, E., and Mappes, T. 2002. Hormonal manipulation of offspring number: maternal effort and reproductive costs. *Evolution* 56: 1530–1537.

Ptacek, M. B. 1996. Interspecific similarity in life-history traits in sympatric populations of gray treefrogs, *Hyla chrysoscelis* and *Hyla versicolor*. *Herpetologica* 52: 323–332.

Rafinska, A. 1991. Reproductive biology of the fire-bellied toads, *Bombina bombina* and *B. variegata* (Anura: Discoglossidae): egg size, clutch size and larval period length differences. *Biol. J. Linnean Soc.* 43: 197–210.

Regard, E., Taurog, A., and Nakashima, T. 1978. Plasma thyroxine and triiodothyronine levels in spontaneously metamorphosing *Rana catesbeiana* tadpoles and in adult anuran amphibia. *Endocrinology* 102: 674–684.

Robertson, J. C., and Kelley, D. B. 1992. Gonadal and laryngeal development in hypothyroid tadpoles. *Am. Zool.* 32: 86A.

Rose, C. S. 1996. An endocrine-based model for developmental and morphogenetic diversification in metamorphic and paedomorphic urodeles. *J. Zool. London* 239: 253–284.

Rosenblum, E. B., Hoekstra, H. E., and Nachman, M. W. 2004. Adaptive reptile color variation and the evolution of the MC1R gene. *Evolution* 58: 1794–1808.

Rot-Nikcevic, I., and Wassersug, R. J. 2004. Arrested development in *Xenopus laevis* tadpoles: how size constrains metamorphosis. *J. Exp. Biol.* 207: 2133–2145.

Shaffer, B. M. 1963. The isolated *Xenopus laevis* tail: a preparation for studying the central nervous system and metamorphosis in culture. *J. Embryol. Exp. Morphol.* 11: 77–90.

Shi, Y., Wong, J., Puzianowska-Kuznicka, M., and Stolow, M. 1996. Tadpole competence and tissue-specific temporal regulation of amphibian metamorphosis: roles of thyroid hormone and its receptors. *Bioessays* 18: 391–399.

Shi, Y.-B. 1999. *Amphibian Metamorphosis: From Morphology to Molecular Biology*. John Wiley and Sons Inc., New York, pp. 1–288.

Swingle, W. W. 1918. The acceleration of metamorphosis in frog larvae by thyroid feeding, and the effects upon the alimentary tract and sex glands. *J. Exp. Zool.* 24: 521–543.

Tata, J. R. 1996. Metamorphosis: an exquisite model for hormonal regulation of post-embryonic development. Extracellular regulators of differentiation and development Biochemical Society Symposium, Vol. 62, pp. 123–136.

Truman, J. W., and Riddiford, L. M. 1999. The origins of insect metamorphosis. *Nature* 401: 447–452.

Ugurtas, I. 1995. Turkeye'deki *Pelobates syriacus* Boettger 1889 (Anura, Pelobatidae)'un Taksonomi, Biyoloji ve Dagilisi Uzerine Arastirmalar. *Turkish J. Zool.* 19: 123–145.

Weber, G. M., Farrar, E. A., Tom, C. K. F., and Grau, E. G. 1994. Changes in whole-body thyroxine and triiodothyronine concentrations and total content during early development and metamorphosis of the toad *Bufo marinus*. *Gen. Comp. Endocrinol.* 94: 62–71.

White, B. A., and Nicoll, C. S. 1981. Hormonal control of amphibian metamorphosis. In L. I. Gilbert and E. Frieden (eds.). *Metamorphosis*. Plenum Press, New York, pp. 363–396.

Wright, A. H., and Wright, A. A. 1949. *Handbook of Frogs and Toads of the United States and Canada*. Cornell University Press, Ithaca, NY, USA, pp. 640.

Yen, P. M. 2001. Physiological and molecular basis of thyroid hormone action. *Physiol. Rev.* 81: 1097–1142.

Zera, A. J., Potts, J., and Kobus, K. 1998. The physiology of life-history trade-offs: experimental analysis of a hormonally induced life-history trade-off in *Gryllus assimilis*. *Am. Nat.* 152: 7–23.