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Effects of Growth History and Exogenous Thyroxine on Size and Age at Metamorphosis in the Toad *Bufo americanus*

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By using food rations and thyroxine supplements, I manipulated the growth and differentiation of tadpoles of the toad *Bufo americanus* to test the hypothesis that the plasticity in metamorphic timing can be lost. A significant food effect indicated that tadpoles that grew rapidly during the middle period of the experiment metamorphosed earlier than slow-growing tadpoles. The changes in growth induced early and late in this experiment did not influence metamorphic timing. There was a significant thyroxine effect: all tadpoles treated with thyroxine metamorphosed early. All thyroxine-treated tadpoles metamorphosed at the same time, indicating that differentiation at the time of thyroxine supplementation was independent of growth rate. A food-by-thyroxine interaction provided evidence that the growth rate/differentiation antagonism may have been active at least during the middle of the experiment. This suggests that the growth rate/differentiation antagonism is decoupled or overridden during later stages of larval development. This result implies limits to metamorphic plasticity and is consistent with a fixed-rate model of amphibian metamorphosis.

AMPHIBIAN metamorphosis is an endocrine event that can have profound ecological consequences (e.g., Wilbur, 1987; Wilbur and Fauth, 1990; Ryan and Semlitsch, 1998). In theory, an amphibian should delay metamorphosis when larval growth opportunities are favorable (e.g., Wilbur and Collins, 1973; Werner, 1986; Rowe and Ludwig, 1991). The result is the potential to maximize size and time of metamorphosis. In some cases, amphibians that are large at metamorphosis are younger and larger at first reproduction (Smith, 1987; Semlitsch et al., 1988). Thus, there should be direct selection for this ability to optimize metamorphic timing to balance the benefits of rapid larval growth with the risks associated with an unpredictable larval environment.

This ecological view of amphibian metamorphosis seems consistent with the endocrinological perspective. The timing of amphibian metamorphosis is regulated by the hypothalamus–pituitary–thyroid axis. When endogenous levels of thyroid hormones (e.g., thyroxine) are high, metamorphosis is initiated (Bern et al., 1967; Etkin and Gona, 1967, Brown, 1997). The action of thyroxine can be influenced to some extent by growth rate (Moriya, 1983). Larvae treated with prolactin grow rapidly and delay metamorphosis, meaning that an antagonism may exist between growth rate and rate of differentiation (Etkin and Gona, 1967; Moriya, 1983; Rosenkilde and Ussing, 1996). Presumably, rapid larval growth will inhibit the initiation of metamorphosis. If a larval amphibian exhibits tween growth and differentiation, then it should have the ability to "detect" a deteriorating environment (i.e., growth and survival potential are diminishing) throughout the larval period. Larvae that experience reduction in growth rate should exhibit less suppression of thyroid hormones, with the ecological result of earlier metamorphosis (Moriya, 1983; Wilbur and Collins, 1973).

Although there is abundant evidence that variation in growth rates in tadpoles and larval salamanders results in variation in metamorphic timing (e.g., Alford and Harris, 1988; Hensley, 1993; Ryan, 2000), the relationship between recent growth history and control of metamorphosis is not resolved. Hensley (1993) suggested that metamorphic flexibility may be lost in the later stages of larval development. For example, metamorphic timing of several species of tadpoles is unaffected by changes in growth rate occurring late in the larval period (e.g., after Gosner stage 35; Hensley, 1993; Leips and Travis, 1994; Beachy et al., 1999). This implies a decoupling of the antagonism between growth rate and rate of differentiation. In contrast, models that predict (or depend on) the retention of metamorphic flexibility (e.g., Wilbur and Collins, 1973; Werner, 1986; Rowe and Ludwig, 1991) imply the persistence of the antagonism.

Tests of the hypothesis that growth history can affect metamorphic timing have been developed using the results of experimental growth rate manipulations (via food treatments, e.g., Alford and Harris, 1988). One can also manipulate rate of differentiation (via exogenous

thyroxine treatment; e.g., Beachy et al., 1999; Ryan, 2000). I used both approaches in a multifactorial experiment. I tested the hypothesis that metamorphic flexibility can be lost in the American toad, *Bufo americanus*. I manipulated the rate of larval growth (via food treatments) with the expectations that, so long as the minimal size requirement (Wilbur and Collins, 1973) is met, (1) tadpoles that experience a decrease in growth rate should initiate metamorphosis and (2) tadpoles that experience an increase in growth rate should delay metamorphosis. In addition, I accelerated differentiation of half of the tadpoles (via exogenous thyroxine treatment) with the expectation that the increase in thyroxine should accelerate metamorphosis to a greater degree in slow-growing tadpoles. Deviations from these expectations would mean that metamorphic flexibility is lost and would suggest that the growth rate/differentiation antagonism has been decoupled.

Materials and Methods

I collected several hundred *B. americanus* eggs from multiple females from a temporary pool in Dubuque County, Iowa, on 12 June 1997. Eggs were maintained in aerated, aged (3 days), dechlorinated tapwater. Hatchlings emerged on 16 June, and 240 individuals were haphazardly chosen and placed in individual 375 mL plastic cups with 250 mL of aged, dechlorinated tapwater.

Food treatments simulated conditions of (1) constant growth and (2) changing growth opportunity. Half of the 240 tadpoles received high food rations at the beginning of the experiment; the remainder received low food rations. After 15 days, the rations of 80 tadpoles were switched (40 tadpoles each from the high food and low food treatments). At Gosner stages 32–35 (Gosner, 1960), the rations of 80 more tadpoles were switched (approximately 26–29 days posthatching). When the second food switch occurred, thyroxine supplementation began. The result was 12 treatments, each replicated 20 times: six food treatments that did not receive thyroxine supplements during the final feeding period (HHH, HLL, HHL, LLL, LHH, LLH) and six that did (HHHt, HLLt, HHLt, LLLt, LHHt, LLHt). In the most simple of natural conditions, conditions for larval growth switch only once (from good to bad with increasing density or bad to good with decreasing density). More complex switching regimes were not explored.

Thyroxine treatment was a 250 μl aliquot of 3,4 thyroxine (Sigma) that, when added to the cup, brought the exogenous concentration to 6×10^{-9}M (5 ppb). I chose this level in an attempt to provide only a small supplement of thyroxine. Endogenous thyroxine concentration in premetamorphic *Rana pipiens* tadpoles is 20–50% of my exogenous supplement; levels during prometamorphosis are 200–600%; and during metamorphic climax, levels are 50-fold greater than the supplements provided to the tadpoles in this experiment (Steinmetz, 1954; Etkin, 1968). Endogenous thyroxine in metamorphosing salamanders is also much higher than the exogenous supplements we provided (Larras-Regard et al., 1981; Alberch et al., 1986). Because thyroxine is soluble only in basic solution, we added a 250 μl aliquot of basic solution minus thyroxine to all nonthyroxine treatments. Thyroxine and control aliquots were added when water was changed. Changes in pH were not detectable in the cups after addition of control and thyroxine aliquots.

Treatments were initiated when tadpoles were placed in cups. The experimental design was a complete randomized block design (six feeding groups × two thyroxine groups = 12 treatments placed in 20 replicate blocks). A ration of either 25 mg (= H) or 12 mg (= L) of a finely ground mixture (1:1) of TetraMin flake fishfood and Heinold Show Formula rabbit chow was provided to each tadpole every 3 days. Cups were rinsed and water replaced with fresh water prior to each feeding.

Every 10 days, tadpoles were blotted dry, weighed to the nearest milligram, and returned to their cup. Upon metamorphosis [Gosner stage 42; defined as the emergence of a forelimb (Gosner, 1960)], animals were again weighed and duration of larval period was recorded.

Growth profiles were examined visually to ensure that food treatment had desired effects on growth rate. Size at metamorphosis and duration of the larval period were analyzed with a multivariate analysis of variance (MANOVA); univariate ANOVAs of each variable were conducted only after a significant MANOVA result. To reduce heteroscedasticity, data on duration of larval period were log-transformed. Data for metamorphic size met criteria for analyses. Preliminary analyses indicated nonsignificant block effects; thus data were analyzed in a full-factorial design. The significance criterion in all analyses was set as $\alpha = 0.05$.

Results

Growth.—Variation in growth rates corresponded to treatment groups' feeding regimes, and

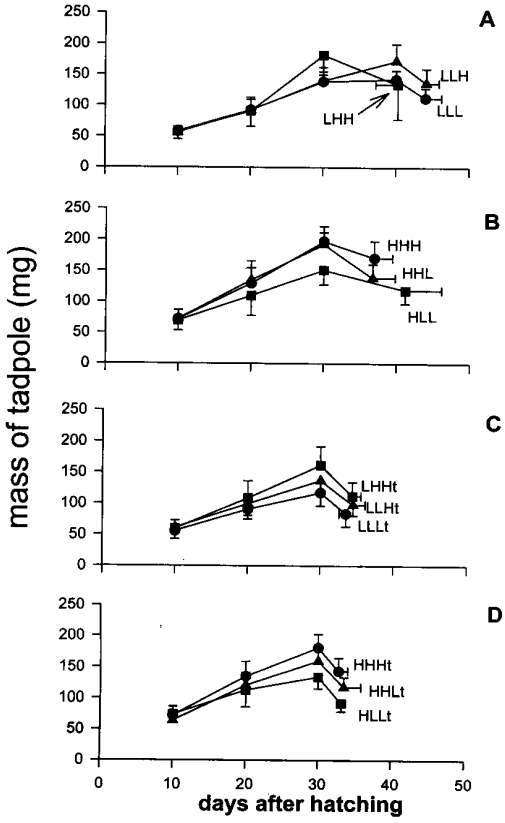

Fig. 1. Growth profiles of the 12 treatment groups. Each point represents the mean mass determined every 10 days. Profiles terminate at the mean metamorphic date for each treatment. Letters refer to feeding regimen for each feeding period; H = high food, L = low food. A "t" indicates groups that received thyroxine supplements when tadpoles reached Gosner stages 32–35 (approximately 26–29 days posthatching). The first food switch occurred after 15 days; the second switch was implemented when tadpoles reached Gosner stages 32–35. Bars represent "+" or "-" 1 SE. (A) Begun on low food (no thyroxine supplements), (B) begun on high food (no thyroxine supplements), (C) begun on low food (received thyroxine supplements), and (D) begun on high food (received thyroxine supplements).

growth of thyroxine and nonthyroxine sister treatments were similar until the application of thyroxine aliquots (Fig. 1). Afterward, thyroxine groups experienced decreases in mass because of accelerated metamorphosis and presumably also because of the dehydrating effects of thyroxine (Moriya, 1982; Moriya and Dent, 1986).

Duration of larval period.—The feeding regime had significant effects on timing of metamorphosis (food effect; Table 1). In general, tadpoles growing rapidly during the second feeding period (HHH, HHL, LHH) metamorphosed earlier than those growing slowly during this period. Neither early nor most recent growth history had significant effects on duration of larval period (e.g., HHH and HHL tadpoles metamorphosed at the same time; Fig. 2).

Not surprisingly, thyroxine application had a significant effect on the timing of metamorphosis (Thyroxine effect; Table 1). All thyroxine groups metamorphosed at the same time regardless of growth history (Fig. 2). The degree of thyroxine-induced acceleration was dependent on food regime (food × thyroxine effect; Table 1). When comparing the acceleration of metamorphosis between thyroxine and nonthyroxine sister treatments (e.g., HHH vs HHHt), it appears that groups that were growing rapidly during the second period of the experiment experienced less thyroxine-induced acceleration than groups growing slowly during this period (e.g., the difference between mean larval period of HHH and HHHt is less than the difference between LLL and LLLt; Fig. 2).

Metamorphic size.—Only the main effects produced significant effects on metamorphic size (Table 1). Food treatments produced expected results (e.g., HHH > HLL, LLH > LLL, HHHt > HHLt). When reared under similar feeding regimes, the thyroxine treatment resulted in a decrease in metamorphic mass, a correlated effect of metamorphosis. Later metamorphosis in the nonthyroxine treated tadpoles allowed for additional growth and thus larger metamorphic size.

Discussion

Regardless of the lack of a clear understanding of environmental effects on the amphibian endocrine system, there exists a large literature concerning the tissue- and organ-level aspects of metamorphosis. The neuroendocrine system (in particular, the hypothalamus and pituitary gland) controls metamorphosis by regulating the activity of the thyroid and interrenal glands. The hypothalamus secretes corticotropin-releasing hormone (Denver, 1996) that travels to the pituitary via the median eminence (Etkin and Sussman, 1961; Eagleson and Malacinski, 1986), where it stimulates the pituitary to produce thyroid-stimulating hormone causing the thyroid to produce thyroid hormones (including thyroxine). Thyroid hormones increase in concentration, causing metamorphosis.

Larval development is characterized by the

TABLE 1. SUMMARY OF MANOVA AND ANOVAS ON DURATION OF LARVAL PERIOD AND METAMORPHIC SIZE FOR LARVAL *Bufo americanus*. Data on duration of larval period were log-transformed for these analyses. Mean squares for larval period are $\times 10^{-3}$.

	Multivariate analysis		Univariate analyses				
			Duration of larval period			Metamorphic size	
Source	df	Wilks' λ	df	MS	*F*	MS	*F*
Food	10,250	0.376*	5	8.29	12.95*	12,423.54	25.09*
Thyroxine	2,126	0.240*	1	191.25	298.62*	35,457.66	71.60*
Food × thyroxine	10,252	0.708*	5	5.16	8.06*	834.56	1.69
Error			127	0.64		495.19	

* $P < 0.001$.

maturation of the median eminence (Eagleson, 1976; Eagleson and Malacinski, 1986), which results in greater secretion of thyroid hormones. Thus, a positive feedback loop develops wherein elevated thyroxine levels stimulate increased production of thyroid hormones. However, in certain tissues/organs, the action of the thyroid hormones can be inhibited by exogenous supplements of prolactin, a hormone that has been hypothesized to affect growth in larval amphibians (Etkin, 1968). Furthermore, Etkin (1968) hypothesized that the elevated production of thyroxine also acts to inhibit pituitary secretion of prolactin, thereby inhibiting growth in the metamorphosing larva (or allowing thyroid hormones to have greater impact in slow-growing larvae). These supplements of prolactin produce pharmacological effects (rather than physiological effects), and prolactin is not the amphibian larval growth hormone (Buckbinder and Brown, 1993; Brown, 1997). Nonetheless, Etkin's suggestion of an antagonism between growth rate and rate of differentiation remains consistent with the adaptive view of metamorphosis wherein a larva that experiences a decrease in growth rate will initiate metamorphosis (Wilbur and Collins, 1973; Werner, 1986; Rowe and Ludwig, 1991).

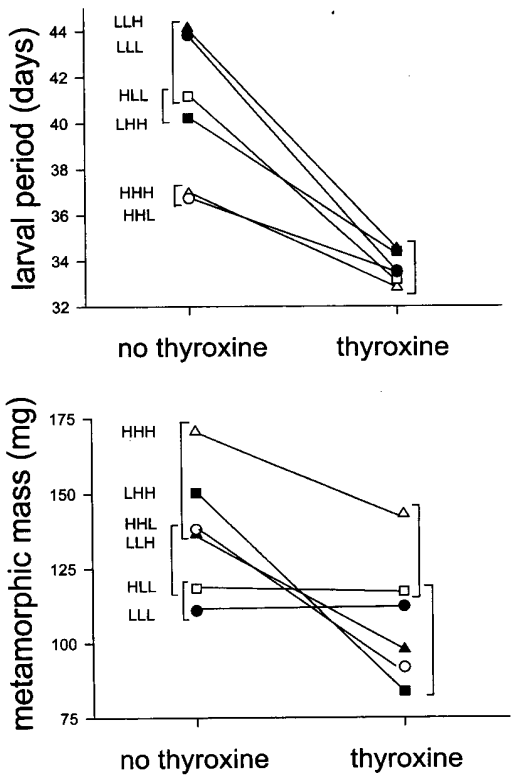

Fig. 2. Summary of a posteriori Tukey's HSD contrasts. Brackets indicate groups that did not differ significantly ($\alpha = 0.05$). Lines connect nonthyroxine groups with thyroxine sister treatments to emphasize the effects of thyroxine-induced acceleration of metamorphosis. Refer to Figure 1 for explanation of codes.

My results indicate that rate of differentiation was not associated with tadpole growth rate: most recent growth history did not affect timing of metamorphosis and groups growing rapidly at the time of thyroxine application did not experience growth rate-suppression of thyroxine action. Coupled with a similar result for *Hyla versicolor* (Beachy et al., 1999), these data indicate that this antagonism may not exist during the later stages of amphibian larval development [i.e., late prometamorphosis (Etkin, 1968)]. Alternatively, the thyroxine surge of late prometamorphosis may accelerate differentiation to a degree that may be sufficient to overwhelm growth rate. That the effect of late supplements of thyroxine was little influenced by growth rate supports this view.

There was evidence for an association between growth rate and rate of differentiation during the second feeding period. The thyroxine-induced acceleration of metamorphosis was more dramatic in tadpoles growing slowly dur-

ing this period. Keeping in mind that the thyroxine treatment was initiated in the third period of this experiment, it would appear that residual effects of rapid growth prior to thyroxine treatment persist and can suppress the action of thyroid hormones. If there is an association between growth and differentiation during the middle of the larval period, how can it act to produce adaptive plasticity for the whole organism? Stated differently, is the variation in metamorphic timing that was produced by variation in growth rate during the second feeding period consistent with any ecological model?

The model provided by Wilbur and Collins (1973) suggests that metamorphic flexibility persists throughout the larval period, implying that the antagonism also persists through metamorphosis. My data are not consistent with this idea. Recent growth history did not affect metamorphic timing (e.g., HHH and HHL treated tadpoles did not metamorphose at different times). Hensley (1993) suggested that the Wilbur and Collins model remains useful if a point of "developmental fixation" is invoked: If a larva reaches the point where the metamorphic "decision" is made, then changes in growth after this point fail to affect larval period. Essentially, this means that, at some point during larval differentiation, the hormonal cascade to initiate metamorphosis has begun and cannot be repressed. In this case, if all tadpoles had exceeded the minimal size requirement, then only the earliest food reductions should cause accelerated metamorphosis. HHH and HHL tadpoles metamorphosed earlier than HLL; this result is consistent with Hensley's (1993) modification of the Wilbur and Collins model only if HLL tadpoles were metamorphosing at the minimal size threshold (Wilbur and Collins, 1973). This interpretation is tenable since the LLL-treated tadpoles metamorphosed at the same time and size as HLL tadpoles (i.e., HLL and LLL tadpoles could have been metamorphosing at the minimal size threshold). Because LHH, HHH, and HHL-treated tadpoles did not differ in body size at metamorphosis, it is possible that all these groups were metamorphosing at the maximal size threshold and earlier than other tadpoles. LLH-treated tadpoles, however, did not reach this maximal size yet metamorphosed despite no decrease in growth rate and despite being larger than a possible minimal size threshold (i.e., the size attained by LLL and HLL tadpoles). Whether the Wilbur and Collins (1973) model, including Hensley's (1993) modification, is sufficient to predict metamorphosis remains unclear.

As an alternative, the "dynamic allocation" model of Leips and Travis (1994) suggests that the allocation of energy to growth and development varies over time, and so the influence of larval growth history on differentiation varies. At early ages, allocation is primarily to differentiation. After some time threshold is reached, metamorphic timing has been fixed, and energy is allocated primarily to growth. The result would be that late increases in growth affect only size, not age at metamorphosis. Only growth prior to the time threshold affects metamorphic timing: Rapid growth prior to the final food switch accelerates differentiation (earlier metamorphosis of HHH, HHL, and LHH groups), and slow growth prior to the final food switch retards differentiation (later metamorphosis of LLL, LLH, and HLL groups). Because rate of differentiation is set, late additions of thyroxine should have equal impact, an interpretation that is consistent with my data (i.e., all thyroxine groups metamorphosed at the same time).

Amphibian ecologists have long appreciated that, to understand the community ecology and life-history evolution of the complex life cycle, it is critical to understand the conditions that favor the evolution and maintenance of adaptive plasticity in metamorphosis (Newman, 1992). This requires an understanding of the endocrine control of this plasticity. Given that the primary means of explaining the plasticity in metamorphic timing has always appealed (explicitly or implicitly) to the differentiation/growth rate antagonism, one needs to be resigned to the idea that a larval amphibian may not be always able to detect favorable/unfavorable growth conditions to delay/accelerate metamorphosis. Such an idea is not foreign to the biologists who have found drying ponds with tadpoles stranded in them.

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