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# Effects of Developmental and Growth History on Metamorphosis in the Gray Treefrog, *Hyla versicolor* (Amphibia, Anura)

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*ABSTRACT* In ecological models, the timing of amphibian metamorphosis is dependent upon rate of larval growth, e.g., tadpoles that experience a decrease in growth rate can initiate metamorphosis early. Recent authors have suggested that this plasticity may be lost at some point during the larval period. We tested this hypothesis by exposing groups of tadpoles of the gray treefrog, *Hyla versicolor*, to different growth schedules. In endocrine models, metamorphosis is dependent on thyroxine levels and thyroxine is antagonized by prolactin (amphibian larval growth hormone), consistent with the idea that a rapidly growing tadpole can delay metamorphosis. Thus, we also manipulated the rate of development by supplementing or maintaining natural thyroxine levels for half of the tadpoles in each growth treatment. All tadpoles that received thyroxine supplements metamorphosed at the same time regardless of growth history. They also metamorphosed earlier than tadpoles not treated with thyroxine. Tadpoles not given thyroxine supplements metamorphosed at different times: those growing rapidly during day 15–34 metamorphosed earlier than tadpoles growing slowly. Growth rate before day 15 and after day 34 had no effect on metamorphic timing. The difference in larval period between these rapidly growing tadpoles and their sisters given thyroxine treatments was less than the same comparison for tadpoles that grew slowly during the same period. This apparent prolactin/thyroxine antagonism did not exist after day 34. These results are consistent with the hypothesis of a loss of plasticity in metamorphic timing. *J. Exp. Zool. 283:522–530, 1999.* 

Given the importance of phenotypic plasticity as an adaptation to variable environments (Bradshaw, ’65; Levins, ’68; Via, ’87; Stearns, ’89), it seems critical to uncover the mechanisms by which adaptive plasticity is controlled and limited (Newman, ’92). For example, the larval stage of most amphibians appears to be an opportunity to take advantage of transient growth opportunities in ephemeral, productive habitats (Wassersug, ’75; Wilbur, ’80; Newman, ’94). Because metamorphosis represents an escape from these habitats, amphibians should initiate metamorphosis whenever conditions become too hostile (e.g., pond-drying). However, amphibians (especially tadpoles) commonly become trapped (and die) in the larval habitat before metamorphosis is possible (Wilbur, ’80; Newman, ’92).

Metamorphosis in these animals is thought to represent a switchpoint during the life cycle that is designed to maximize the growth-to-mortality risk ratio in both the larval and adult environments (Werner and Gilliam, ’84; Werner, ’86; Rowe and Ludwig, ’91). Because the larval environment is often highly variable (e.g., pond-drying or arrival of predators is unpredictable), a larva that maintains a plastic response in timing of and/or size at metamorphosis may have higher fitness than one with fixed metamorphic parameters. Wilbur and Collins (’73) hypothesized that amphibian larvae respond adaptively to resource variation by initiating metamorphosis when a larva experiences a decrease in growth. This response to a deteriorating environment (e.g., increasing density of competitors, presence of predators) is suggested to occur between a minimal size required to initiate metamorphosis and a maximal size when metamorphosis is obligatory.

There is abundant evidence that larval growth history has significant effects on timing of metamorphosis (Collins, ’79; Semlitsch and Caldwell, ’82; Semlitsch and Gibbons, ’85; Alford and Harris, ’88; Newman, ’89; Skelly and Werner, ’90;

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Pfennig et al., '91; Hensley, '93; Leips and Travis, '94). However, several authors have suggested that metamorphic flexibility is lost during later stages of the larval period; thus, there is a limit to this capacity for adaptive response (Smith-Gill and Berven, '79; Travis, '84; Hensley, '93; Leips and Travis, '94).

From a developmental standpoint, the initiation of metamorphosis is under control of the hypothalamus-pituitary-thyroid axis (Gilbert, '88). Stress situations (e.g., increasing density, decreasing food availability) cause hypothalamic production of corticotropin-releasing hormone (CRH) which travels to the pituitary via the median eminence (Denver, '97). CRH stimulates the pituitary to release thyroid stimulating hormone which causes the thyroid to produce triiodothyronine and thyroxine, the principal hormones involved in metamorphosis (Bern et al., '67; Etkin and Gona, '67; Etkin, '68; Rosenkilde and Ussing, '96). In the growing larva, the action of the thyroid hormones is inhibited by the growth hormone prolactin (Moriya, '83), which is also produced by the pituitary. As the concentrations of triiodothyronine and thyroxine increase, feedback loops stimulate more production of thyroid hormones and inhibit prolactin secretion. Thus a fast-growing larva (i.e., possessing a high prolactin/thyroid hormone ratio) should initiate metamorphosis later than a slow-growing larva, consistent with the predictions of the Wilbur-Collins ('73) model.

Several studies found that variation in growth rate induced late in the larval period had no effect on metamorphic timing (Hensley, '93; Leips and Travis, '94). Any model that suggests a loss of metamorphic flexibility (herein referred to as "Loss" models) implies a decoupling of the prolactin-thyroxine relationship. The Wilbur-Collins ('73) model demands that, if metamorphic flexibility is always available, the prolactin-thyroxine relationship remains antagonistic throughout the larval period.

We tested for the persistence of metamorphic flexibility by manipulating growth and developmental rates in the tadpoles of the gray treefrog, *Hyla versicolor*. Our null hypothesis was that changing the rate of growth and/or development will not affect duration of the larval period. Controlling the rate at which a tadpole grows (by food treatments) and develops (by thyroxine treatment) allowed us to determine when and if phenotypic plasticity in metamorphic timing is lost, thereby providing a test of the validity of the Wilbur-Collins model versus "Loss" models.

## MATERIALS AND METHODS

The gray treefrog, *Hyla versicolor*, occurs in North America from southeast Manitoba south to east Texas, and east to the Atlantic coast. In the field, *H. versicolor* is distinguished from its cryptic sister species, *Hyla chrysoscelis*, by the mating call of the males (Conant and Collins, '91). They are summer breeders and utilize ephemeral ponds that are filled by rains.

Eggs of *H. versicolor* were collected from a temporary pond in Dubuque County, Iowa on May 20, 1996. The eggs were taken to the laboratory and placed in an aerated aquarium with aged, dechlorinated tap water. The eggs hatched after four days (May 24). After hatching, 240 individual tadpoles were placed singly in plastic cups in 250 mL of aged, dechlorinated tap water at 22 ± 1°C. Each tadpole was randomly assigned to one of 20 spatial blocks. Each block consisted of 12 cups, one for each treatment. The blocks were placed on four tables and were randomly assigned to a new position every 10 days. The 12 cups within each block were randomly assigned to each of the 12 treatment groups.

We used a multifactorial design to manipulate growth and developmental rate in the tadpoles. Two factors were established: initial food abundance (high or low) followed by a switch in food availability (no switch, early switch, or late switch), and a supplement of metamorphic hormone (thyroxine or no thyroxine). Thus, a total of 12 treatment groups ($6 \times 2 = 12$) were established, with 20 tadpoles per treatment (Table 1).

Food treatments were designed to simulate conditions of (a) constant growth and (b) changing growth opportunity. According to the Wilbur-

*TABLE 1. Summary of treatment groups of* Hyla versicolor[1]

| Treatment code[2] | Begun on | Early switch | Late switch | Thyroxine |
|---|---|---|---|---|
| HHH | high food | no | no | no |
| HHL | high food | no | yes | no |
| HLL | high food | yes | — | no |
| LLL | low food | no | no | no |
| LLH | low food | no | yes | no |
| LHH | low food | yes | — | no |
| HHHt | high food | no | no | yes |
| HHLt | high food | no | yes | yes |
| HLLt | high food | yes | — | yes |
| LLLt | low food | no | no | yes |
| LLHt | low food | no | yes | yes |
| LLHt | low food | yes | — | yes |

[1]The early switch occurred after 15 days; late switch after 34 days when most tadpoles had attained Gosner stage 34.
[2]H = high; L = low; t = thyroxine-treated groups.

Collins ('73) model, tadpoles that experience constant growth (fast [HHH] or slow [LLL]) should metamorphose at the maximal size threshold (achieved later by the slow growers). Those tadpoles that experience an increase in growth rate (LHH and LLH) should also metamorphose at the maximal size threshold, but at an earlier date than the larvae growing at a constant slow rate. Tadpoles that experience a decrease in growth rate (HHL and HLL) should initiate metamorphosis soon after the reduction in growth. In addition, treatment with thyroxine should accelerate metamorphosis in a manner that is dependent upon growth rate: slow-growing tadpoles treated with thyroxine should initiate metamorphosis earlier than fast-growing tadpoles treated with thyroxine (e.g., LLLt vs. LLHt, HLLt vs. HHHt). The predictions of the Wilbur-Collins model for the treatment groups are shown in Fig. 1.

Predictions concerning the thyroxine treatments are more speculative given that there are no quantitative data about how growth rate interacts with thyroxine levels.

Food treatments were either 25 mg (high) or 12 mg (low) of a 1:1 mixture of finely ground fish food (TetraMin tropical fish flakes, Blacksburg, VA) and rabbit chow (Heinold Show formula 15–20 rabbit pellets, Kouts, IN), administered every three days. Water was changed prior to each feeding. Thyroxine treatment consisted of a 250 μL aliquot of thyroxine solution that, when added to the 250 mL of water in the cup, brought the thy-

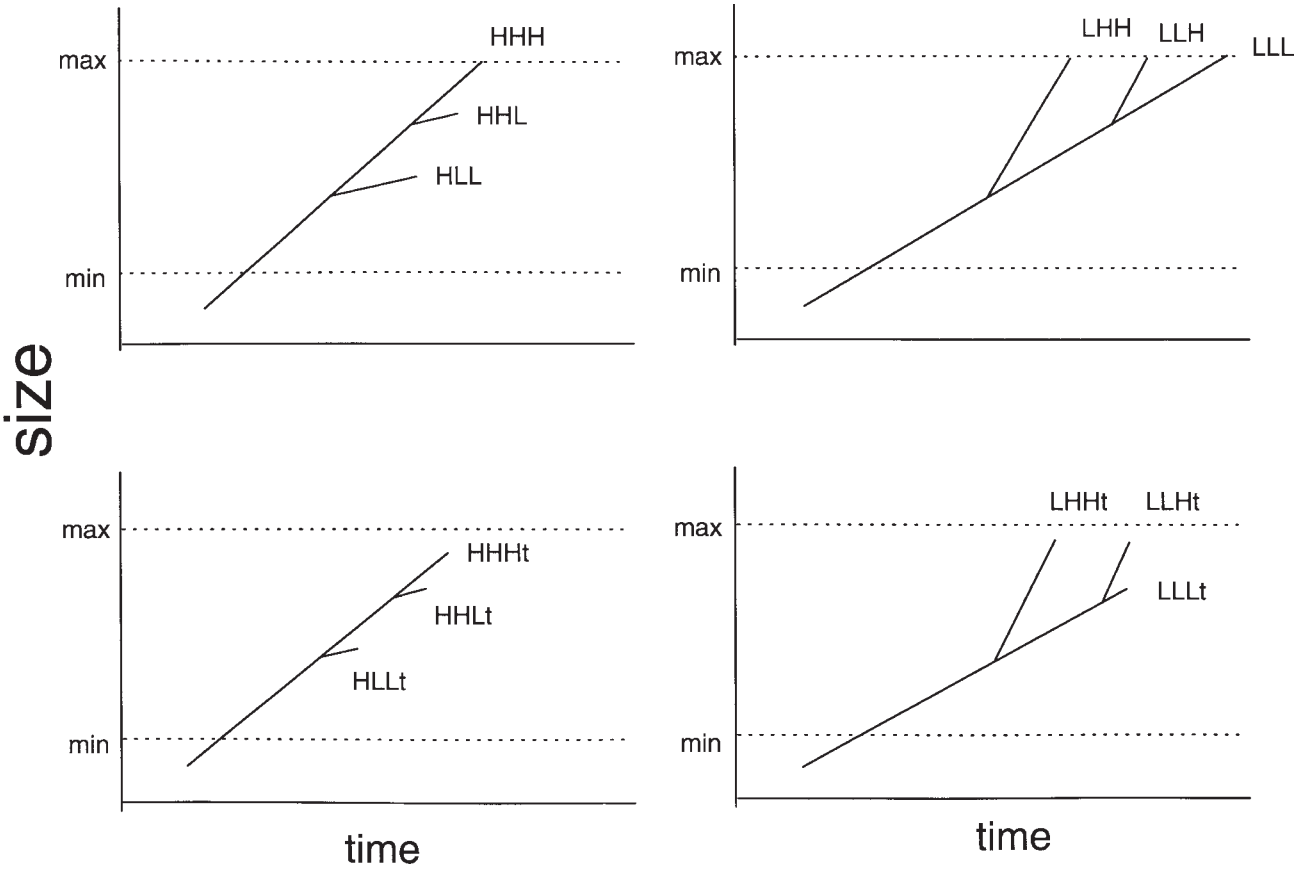


Fig. 1. Predictions of metamorphic timing and size in treatment groups. The Wilbur-Collins model predicts that amphibian larvae that experience a reduction in growth opportunity will initiate metamorphosis. Otherwise, growth should continue until a maximal metamorph size threshold is achieved in order to take advantage of the growth opportunity. The expected results, based on the Wilbur-Collins model, of our treatments are presented above. Treatment codes are given in Table 1. Each growth trajectory terminates at the size and time of metamorphosis. Note that the thyroxine treatments experience differential truncation of the growth trajectory as predicted by the retention of the antagonistic prolactin/thyroxine relationship. For example, the degree of thyroxine-induced acceleration of metamorphosis is predicted to be less for LLHt than for LLLt, due to the larger prolactin/thyroxine ratio in the former. The exact relationship of growth rate and thyroxine is unknown.

roxine to a concentration of 5 ppb ($6 \times 10^{-9}$ M). Because thyroxine is only soluble in basic solution, we added a 250 μL aliquot of the basic solution minus thyroxine to all non-thyroxine treatments. Thyroxine and non-thyroxine (control) aliquots were added when water was changed. Change in pH in the cups was not detectable following addition of thyroxine and control aliquots.

Food treatments were initiated on the first day of the experiment (May 24). Six of the treatment groups were placed on the high-food regime and the other six were given the low-food regime. Four of the groups (two each of the high-food and low-food treatments) were switched to the opposite food regime 15 days after hatching. All tadpoles were at Gosner stage 25 (Gosner, '60) when this switch was made. Four more groups (two each of the high-food and low-food treatments) were switched to the opposite food regime when the majority of tadpoles had attained Gosner stage 34 (June 27). The timing of food level changes was based on similar experiments (e.g., Alford and Harris, '88; Hensley, '93). The remaining four groups had constant food levels (two high and two low). Thyroxine treatments began when the second food switch was begun (i.e., June 27). Six of the feeding treatments were given thyroxine aliquots during each water change, whereas control aliquots were given to the remaining six groups.

We weighed tadpoles every 10 days after hatching until metamorphosis occurred. Tadpoles were removed from cups, blotted to remove excess water, and weighed to the nearest mg. Cups were checked daily for metamorphosing tadpoles. Duration of larval period was defined as the number of days from hatching to the emergence of at least one forelimb (Gosner stage 42). Forelimbs emerge fully developed, and so provided a discrete indicator of metamorphosis. Upon forelimb emergence, individuals were weighed (= metamorphic size) and returned to the site of collection.

Data met the assumptions for analysis of variance. Analyses were performed using SPSS-X (Norusis, '88). The significance criterion was set as $\alpha = 0.05$, and Wilks' lambda was used as the multivariate test statistic.

Data on metamorphic size and duration of the larval period were analyzed with a two-way multivariate analysis of variance (MANOVA). Univariate results were analyzed only if MANOVA indicated significant differences in response vectors among treatments (Morrison, '76). If significant univariate results were obtained, we performed pairwise Tukey's hsd tests to determine which treatment groups were different from one another (Sokal and Rohlf, '81; Day and Quinn, '89).

## RESULTS

### *Growth*

Growth was inspected visually to ensure that food treatment groups differed in growth rate. Following each increase or reduction in food, treatment groups experienced corresponding increases or reductions in growth rate (Fig. 2). Significant variation in tadpole mass existed 10 days after hatching (Low-food: $\bar{x} = 43.0$ mg, SD = 9.1; High-food: $\bar{x} = 46.2$, SD = 9.9; $t_s = 2.28$, df = 181, $P = 0.02$). Treatment groups continued to diverge throughout the experiment (Fig. 2).

Treatment groups exposed to thyroxine experienced an apparent decrease in growth rate relative to non-thyroxine-treated tadpoles (see after day 34; Fig. 2). This is probably due to the dehydrating effects of elevated thyroxine (Moriya, '82; Moriya and Dent, '86).

### *Larval period*

Duration of larval period was significantly influenced by all factors in the analysis (Table 2). Larval periods were longest in tadpoles receiving non-thyroxine treatments that experienced slow growth after the first food switch and before the second switch (Fig. 3). The shortest larval periods were seen in tadpoles receiving thyroxine treatments (Fig. 3).

#### Food

Variation in food regime, translated into variation in growth history, resulted in two clusters of non-thyroxine treatments: LLL, LLH, and HLL metamorphosed significantly later than LHH, HHH, and HHL (Figs. 2 and 3). The common feature of each cluster of treatments was either rapid or slow growth during the middle portion of the experiment (i.e., between day 15 and 34). Most recent growth history had no effect on metamorphic timing (Fig. 3). For example, HHH and LLL metamorphosed at the same time as HHL and LLH, respectively.

#### Thyroxine

There was a clear (and not surprising) reduction in larval period in thyroxine treatments, indicating thyroxine-induced acceleration of metamorphosis (Fig. 3). Mean larval period for all non-thyroxine treated tadpoles was approximately 46 days; mean larval period for those receiving thyroxine

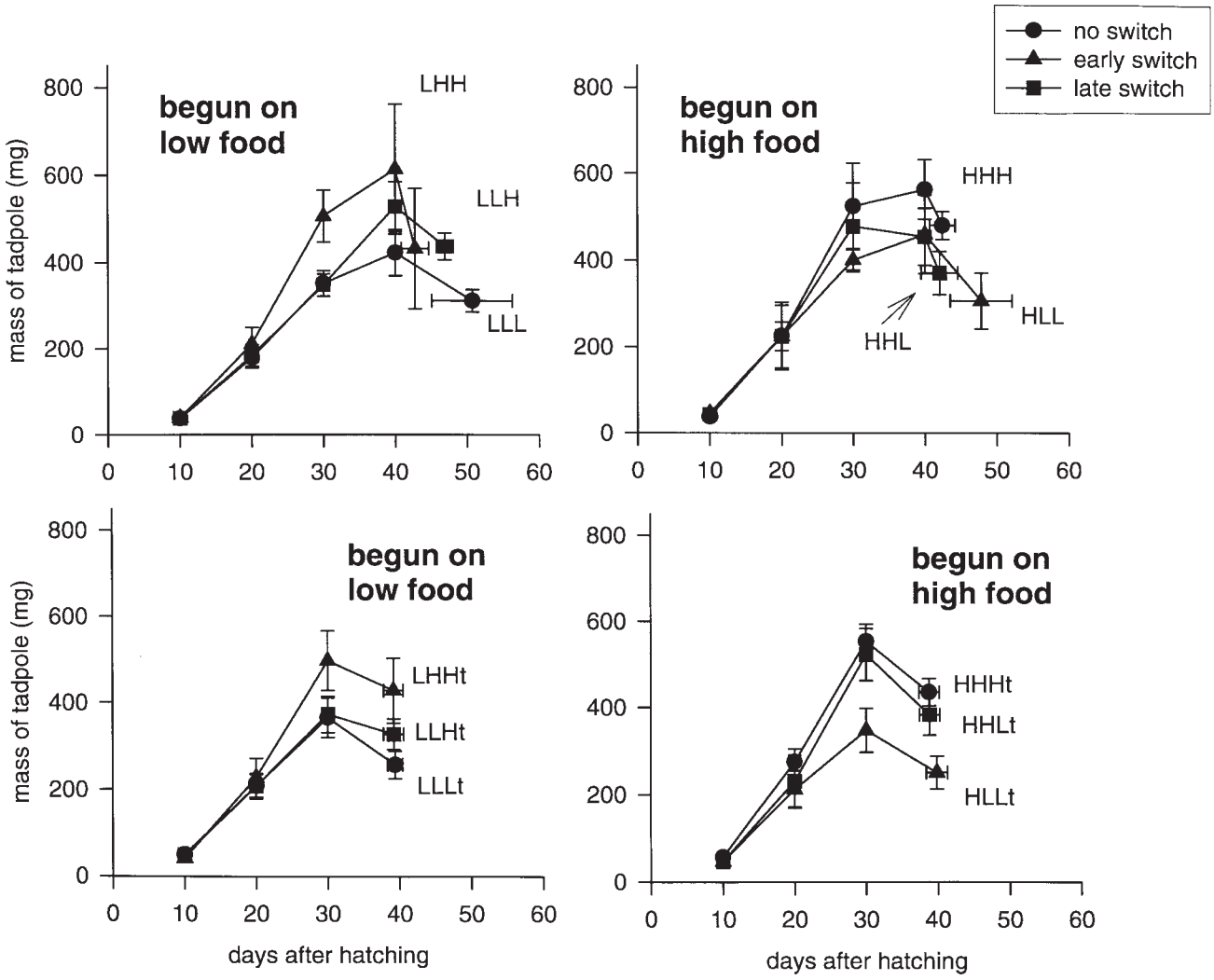


Fig. 2. Growth trajectories of tadpoles. Symbols represent the mean mass of tadpoles in each treatment group. Each growth curve terminates at the mean duration of the larval period for each treatment. Tadpoles were weighed every 10 days. Bars are ±1 standard deviation. Treatment codes are given in Table 1.

treatments was 39 days. All tadpoles receiving thyroxine treatments metamorphosed at the same time (Fig. 3).

**Food × thyroxine**

Food treatments indicated no effect of recent growth history on metamorphic timing (see above) and thyroxine treatment (see above) gave no indication of prolactin-suppression of thyroxine. However, the interaction of these treatments indicated support for the prolactin/thyroxine antagonism (Fig. 3). Tadpoles that grew rapidly from day 15 to day 34 (i.e., LHH, HHH, HHL) experienced less thyroxine-induced acceleration of metamorphosis when compared to thyroxine sister treatments than tadpoles that grew slowly during the same period (e.g., HHH – HHHt < LLL – LLLt). Recent growth history did not have any effect on the degree of thyroxine-induced acceleration of metamorphosis (Fig. 3). This suggests that the antagonism was decoupled after day 34.

### *Metamorphic mass*

Although all treatment factors had significant influence on metamorphic mass (Table 2), their effects were less straightforward than for duration of larval period. These complex results are very likely due to the correlated effect of duration of the larval period, i.e., tadpoles with long larval periods experience more opportunity for growth.

*TABLE 2. Summary of MANOVA of metamorphic size and duration of the larval period of* Hyla versicolor[1]

| | MANOVA | | ANOVA[2] | | | |
|---|---|---|---|---|---|---|
| | | | Larval period | | Metamorphic mass | |
| Source | Wilks' λ | *P* | *F* | *P* | *F* | *P* |
| Food (F) | 0.21 | <0.001 | 14.2 | <0.001 | 62.17 | <0.001 |
| Thyroxine (T) | 0.32 | <0.001 | 242.0 | <0.001 | 30.19 | <0.001 |
| F × T | 0.64 | <0.001 | 9.9 | <0.001 | 3.44 | 0.006 |

[1]For the multivariate statistics, df = 10,256. For the univariate statistics, df = 5,129 (for Food and F × T) or df = 1,129 (for Thyroxine). The univariate statistics report *P* values.
[2]For larval period, the residual mean square = 4.75; for metamorphic mass, the residual mean square = 2166.78.

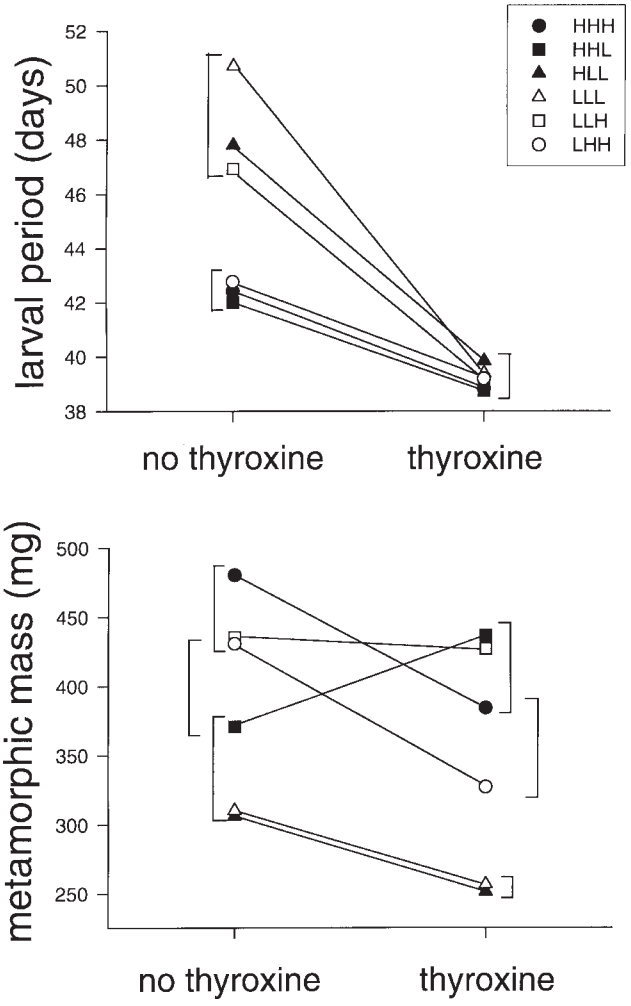


Fig. 3. Results of multiple comparison tests on duration of larval period and metamorphic mass. Brackets indicate means not significantly different by Tukey's hsd tests. Treatment codes are given in Table 1. Lines connecting tadpoles not receiving thyroxine treatments with those receiving thyroxine treatments are drawn to emphasize the thyroxine-induced variation in larval period and metamorphic mass.

### Food

Tadpoles that experienced slow growth after day 15 (i.e., LLL and HLL) metamorphosed at smaller sizes than the rest of the treatments (Fig. 3). Growth opportunities prior to day 15 apparently had little effect on metamorphic mass. Tadpoles that metamorphosed at larger sizes were exposed to an enhanced growth opportunity (a) for the entire experiment, (b) after day 34, or (c) during the first 34 days or after the first 10 days. It appears that the growth opportunities after day 10 were more important in terms of accumulation of mass.

### Thyroxine

Treatment with thyroxine resulted in lower metamorphic mass. This is probably a result of two effects: reduction in larval period and increased dehydration induced by elevated thyroxine levels.

### Food × thyroxine

The non-additive effects of food and thyroxine appear to result from differences among non-thyroxine and thyroxine groups in the reduction of metamorphic mass. Four groups (HHHt, LHHt, LLLt, HLLt) experienced a significant reduction in metamorphic mass as a result of early metamorphosis and increased dehydration when compared to non-thyroxine sister treatments. However, no thyroxine-induced reduction of metamorphic mass occurred in HHLt (mass increased) and LLHt (mass did not change) (Fig. 3). Tadpoles from the HHL treatment delayed metamorphosis relative to the HHLt group, with the result of a decrease in mass during the slow growth period after day 34. Because metamorphosis was accelerated in the HHLt group, the loss in mass during this slow growth period was attenuated. In contrast, LLH tadpoles delayed metamorphosis relative to the LLHt tadpoles, thus enabling these tadpole to take advantage of the late growth opportunity.

## DISCUSSION

The Wilbur-Collins ('73) model has been used to discuss variation in size and time of several life-history transitions in diverse taxa (e.g., metamorphosis in insects (Sweeney and Vannote, '78; Vannote and Sweeney, '80; Blakley, '81) and crustaceans (Twombly, '96), seed set in plants (Willson, '81; Lacey, '86), and maturation in fishes (Policansky, '83; Reznick, '90)). For amphibian metamorphosis, it assumes the persistence of flexibility in metamorphic timing throughout the duration of the larval period. Implied in this assumption is the persistence of the prolactin/thyroxine antagonism.

Our data corroborate previous studies documenting that variation in larval growth history can produce variation in duration of the larval period (Alford and Harris, '88; Newman, '89; Skelly and Werner, '90). However, as suggested by "Loss" models (e.g., Hensley, '93), a point of "developmental fixation" appeared to be attained, upon which later variation in growth rate influenced only size, but not metamorphic timing (i.e., metamorphic flexibility was lost, implying the decoupling of the prolactin/thyroxine antagonism).

"Loss" models vary in their predictions concerning the timing of developmental fixation. Travis ('84) argued that developmental fixation occurs very early in larval development with the result that later changes in growth rate have effects on metamorphic size but not larval period. This implies that any "decision" made about habitat quality occurs early in development. However, effects that occurred between d15 and d34 promoted variation in metamorphic timing. Those tadpoles growing rapidly during the second food regime metamorphosed earlier than those that grew slowly during the same period. Under the tenets of the Wilbur-Collins ('73) model, this could be interpreted as meaning that fast-growers achieved the maximal size threshold earlier and thus metamorphosed earlier. This would mean that all tadpoles metamorphosed at the same size (Fig. 1), but this was not the case (Fig. 3).

The maximal size threshold of Wilbur and Collins ('73) is typically interpreted as the "maximal metamorph size." If this threshold is viewed, rather, as the "maximal tadpole size prior to developmental fixation," then later changes in growth, though not affecting timing, can continue to have effects on size. This variant of the Wilbur-Collins ('73) model was first suggested by Hensley ('93) and is consistent with predictions of the "dynamic allocation" model of Leips and Travis ('94).

As expected, thyroxine treatment accelerated metamorphosis. The persistence of metamorphic flexibility would be expected to result in less thyroxine-induced developmental acceleration in fast-growing tadpoles than in slow-growers, i.e., LLHt tadpoles would experience less thyroxine-induced acceleration of metamorphosis than LLLt tadpoles (Fig. 1). Yet, all tadpoles treated with thyroxine metamorphosed at the same time, suggesting a lack of metamorphic flexibility that is consistent with the "Loss" models.

Because application of thyroxine caused uniform larval periods in tadpoles given thyroxine treatments, this means that the degree of thyroxine action differed depending on growth history (Table 2). The difference in larval periods between thyroxine and non-thyroxine treatments (e.g., LLLt vs. LLL, HLLt vs. HLL) was greatest in tadpoles that experienced slow growth during the middle third of the larval period. The larval periods of tadpoles experiencing rapid growth in the middle portion of the larval period were less reduced compared with tadpoles receiving thyroxine treatments (Fig. 3). This suggests that while developmental fixation may occur late in the larval period, effects of growth during the middle period of the larval period may still inhibit the action of thyroxine. This may result from the greater concentration of growth hormone present in tadpoles that grew rapidly during this period. Prolactin concentration is decreased in slow-growing tadpoles, and the action of thyroid hormones is more effective (Etkin, '68; Gilbert, '88).

The prolactin/thyroxine antagonism is suggested by an inspection of growth during the middle portion of the experiment (Fig. 3). The absence of this antagonism in groups that experienced a late switch in growth documents that the ability to detect a deteriorating habitat was lost (= Loss models) sometime prior to day 34 of this experiment. Tadpoles appear able to "detect" growth opportunities only during the early portion of the larval period. Increases in habitat quality (e.g., reduction in density, increase in food availability) that occur late in the larval period may be beyond the capacity of larval *H. versicolor* to utilize.

The "decision" of when to metamorphose appeared to be determined during the middle portion of the larval period: rapid growth determined early metamorphosis. These data are consistent with the Wilbur-Collins hypothesis only if the maximal size was attained during the period of

developmental flexibility (i.e., between 15 and 34 days). Otherwise, there appears to be little adaptive value to delaying metamorphosis in a poor-quality habitat (i.e., HLL, LLL, and LLH groups metamorphosed later than other groups, a bad strategy in a drying pond). If metamorphic timing was determined in this manner, then, when compared to rapidly-growing tadpoles, slow-growers would allocate more energy towards growth than metamorphosis and thus metamorphose later (Leips and Travis, '94).

Hensley ('93) suggested that the Wilbur-Collins ('73) model can be modified to fit existing data if a point of developmental fixation is incorporated. If the "maximal metamorph size" threshold of Wilbur and Collins ('73) is replaced with "maximal tadpole size prior to developmental fixation" and this threshold is then viewed as occurring prior to metamorphic climax, then Hensley's ('93) contention that "Loss" models are consistent with the Wilbur and Collins ('73) model appears tenable. This threshold (i.e., maximal tadpole size prior to developmental fixation) then becomes a target for selection, and species that occupy unpredictable habitats require the delay of this threshold (Hensley, '93). Further testing with *H. versicolor* and anuran species that occupy even more ephemeral habitats (e.g., *Scaphiopus couchii* [Newman, '94]) should make the constraints on metamorphic flexibility clearer, and can also suggest ways that this kind of phenotypic plasticity can evolve.

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