# Effects of Growth Rate and Temperature on Metamorphosis in *Eurycea wilderae* (Caudata, Plethodontidae, Hemidactyliinae, Spelerpini; Blue Ridge Two-lined Salamander)

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**Abstract -** The southeastern US has an incredibly diverse salamander fauna that contains ~17% of the world's species. Furthermore, there is more variation in salamander life-cycle there than elsewhere—the diversity includes metamorphosing, paedomorphic, and direct-developing species. The most diverse family in the world and in the southeastern US are the salamanders in the family Plethodontidae. I tested the hypothesis that variation in larval-growth history causes variation in metamorphic timing in *Eurycea wilderae* (Blue Ridge Two-lined Salamander), a member of 1 of the 3 lineages of plethodontid salamanders that metamorphose. Larval Blue Ridge Two-lined Salamanders were grown at 2 food levels and at 2 temperatures. Larvae that were fed more food grew faster, but effects on earlier metamorphic timing were minimal (3.84%, 13 d, $P = 0.071$). In contrast, larvae grown at a high temperature metamorphosed an average of 32.98% (55 d) earlier that those grown at a low temperature. In many amphibian species, the timing of metamorphosis is strongly affected by temperature and by variation in food availability. The weak response in timing of metamorphosis to variation in food is consistent with findings for other plethodontids and may be unique among amphibians.

## Introduction

Metamorphosis in amphibians is an important life-history transition because it can directly influence fitness. For example, timing of metamorphosis and larval size at metamorphosis have been shown to affect size and age at sexual maturation (Semlitsch et al. 1988, Smith 1987). Two of the most important factors that affect metamorphic parameters in amphibians are temperature and food (Alford and Harris 1988, Beachy 1995). Low temperature can slow development with the consequence of a longer larval period (Beachy 1995, Hayes et al. 1993, Hickerson et al. 2005). The effects of variation in food availability on larval growth and metamorphosis (and how temperature and food interact) are complex (Beachy 1995, Hickerson et al. 2005, Voss 1993). Current knowledge is generally based on pond-breeding amphibians (Beachy 2001, Beachy et al. 1999, Wilbur and Collins 1973), and in the case of salamanders, the Ambystomatidae and the Salamandridae (Denoël and Joly 2000, Semlitsch et al. 1988). Much less is known about the effect of temperature and food on metamorphosis in the Plethodontidae (Beachy et al. 2017). Most

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plethodontid species that metamorphose use streams as larval habitat (Wake 1966). Given that this family is, by far, the most diverse family of salamanders, it seems essential to understand how food level and temperature influence metamorphosis.

The plethodontid salamander genus *Eurycea* contains 33 species and includes taxa that have either a metamorphic life cycle or larval-form paedomorphosis (Bonett et al. 2014, Ryan and Bruce 1998). In the species that metamorphose, there can be within- and among-population variation in larval life-history characteristics, e.g., duration of the larval period and size at metamorphosis (Bruce 1982, Duellman and Wood 1954, Voss 1993). Intraspecific geographic variation in metamorphic timing and size in species of *Eurycea* has been attributed to several factors, e.g., temperature, stream order and productivity of the larval habitat (Semlitsch 1980, Voss 1993). Nearly all work on the larval life history in species of *Eurycea* has been restricted to field surveys (e.g., Bruce 1988, Ryan 1998) that established metamorphic timing and size (but see Aran et al. 2014, Bonnett and Chippindale 2006, McEntire et al. 2014). There are no laboratory growth experiments on larval *Eurycea* testing for effects of ecological factors on metamorphic timing or size.

Using experimental manipulations, I examined the effects of temperature and food on metamorphic timing and size in *Eurycea wilderae* Dunn (Blue Ridge Two-lined Salamander). The Plethodontidae has 3 lineages with a life cycle that includes a postembryonic metamorphosis: *Hemidactylium scutatum* Temminck & Schlegel in Von Siebold (Four-toed Salamander), the desmognathans, and the spelerpines (Bonnett 2016). No plethodontid salamander studied in larval-growth experiments has shown variation in metamorphic timing despite being grown at different rates via food treatments. These studies have included *Desmognathus ocoee* Nicholls (Ocoee Salamander; Beachy 1995), *Desmognathus quadramaculatus* (Holbrook) (Blackbelly Salamder; Hickerson et al. 2005), and Four-toed Salamander (O'Laughlin and Harris 2000). The spelerpine plethodontids (*Eurycea*, *Pseudotriton*, *Stereochilus*, *Gyrinophilus*) have not been examined. I tested the hypothesis that variation in growth history would result in variation in metamorphic timing in the spelerpine plethodontids by growing larval Blue Ridge Two-lined Salamanders in the laboratory under simulated ecological conditions. I manipulated food and temperature and assayed time and size at metamorphosis. This kind of laboratory growth experiment has been informative in understanding the proximate ecological conditions responsible for influencing life-history parameters in larval amphibians (e.g., Alford and Harris 1988, Beachy et al. 1999, Ryan and Semlitsch 2003).

## Methods

I grew 37 larval Blue Ridge Two-lined Salamanders (either 9 or 10 per treatment) in a 2 X 2 experiment in which I manipulated temperature (low and high) and food level (low and high). I collected larvae on 28 December 2011 from a headwater seepage in Jackson County, NC (31°15'24"N, 83°09'39"W; datum = WGS84). Stream temperature during collection was 7 °C. I placed larvae in 2 plastic containers filled with stream water and leaf litter from the streambed and transported them in a cooler back to the laboratory in Minot, ND. Upon return to the lab, each

I placed each larva in an individual plastic box (15 cm x 15 cm x 5 cm) filled with 200 ml of reverse osmosis (RO) water and 25 g of pebbles (placed in a corner of the box to provide a shelter), and randomly assigned each to 1 of 4 treatment groups. Larval Blue Ridge Two-lined Salamanders from nearby headwater seeps and 1st-order streams at the collection locality were ~7 months old in December (Bruce 1988, Voss 1993).

Larvae were fed *Lumbriculus variegatus* (Müller) (California Blackworm). I obtained the California Blackworms from AquaticFoods.com and stored them in RO water that was changed daily. I fed larvae different numbers of California Blackworms. I fed high-food treatment larvae 4 California Blackworms per week and low-food treatment larvae 1 California Blackworm per week. Larvae in the low-food treatments always consumed the worm within a 24-h period, whereas larvae in the high-food treatments often had worms remaining at the next feeding; in that case, I added only enough worms to bring the total to 4 worms.

I manipulated the temperature by placing larvae in 2 refrigerators (True Company, O'Fallon, MO) equipped with thermostats (Ranco Company, Itasca, IL) and glass doors in a room with lighting set to a 12:12 h light:dark cycle. The high-temperature treatment larvae were kept at 11 °C (± 1 °C ) and the low-temperature treatment larvae were kept at 7 °C (± 1 °C ). I compensated for spatial variation in 2 ways. First, although the larvae were in separate refrigerators, the maximal distance between the groups was 1 m. Second, at randomly assigned intervals, I switched the larvae between refrigerators at the same time that the refrigerator temperature was reset to the other temperature. By the end of the experiment, all larvae had spent an approximately equal number of days in each refrigerator.

The experiment was initiated on 4 January 2012, at which time I measured the snout–vent length (SVL; mm) of all larvae before placing them in the appropriate temperature treatment and providing their first feeding. The next feeding occurred on 12 January and every 7 d thereafter. I again measured body size after 49 d and 68 d, and every 14 d thereafter until all animals had completed metamorphosis. On 16 January and every 14 d thereafter, I removed with a net and placed each larva in a 50-ml beaker of water, rinsed the plastic box and pebbles, refilled the box with 200 ml of fresh RO water that was preconditioned to the appropriate temperature for each treatment, and replaced the larva into the box and placed it back into the appropriate temperature treatment.

Beginning on day 54, I raised the refrigerator temperatures 1 °C every 14 d until temperatures were 16 °C and 12 °C (day 110) in the high- and low-temperature treatments, respectively. These increases mimicked rising spring temperatures at the collection locality. I chose those temperatures because they encompassed the range of natural-temperature variation in headwater streams across the range of Blue Ridge Two-lined Salamander (e.g., Voss 1993). I doubled the food (i.e., 8 worms and 2 worms at each weekly feeding, respectively) when temperature levels reached 16 °C and 12 °C.

To measure body size, I placed larvae in a circular glass dish containing a 20-mm ruler and mounted this dish on a pedestal that allowed for the larva to be photographed from underneath. I downloaded these images to a personal computer

and employed ImageJ software to determine SVL. As each larva completed metamorphosis (defined as closure of gill slits and resorption of labial folds), I recorded the number of days since the initiation of the experiment and metamorphic size (SVL). Animals were not euthanized at the end of the experiment so that they could be used in a continuing growth experiment.

I visually inspected growth profiles (Fig. 1) during the experiment to verify that the food treatments had desired effects, i.e., that larvae grew at different rates. In addition, I evaluated the effects of temperature and food treatments on growth by analyzing larval sizes in a 2 x 2 full-factorial MANOVA and ANOVA that analyzed the effects of temperature and food on the vector consisting of sizes at days 0, 49, 68, and 82. The analysis did not include body size after day 82 because animals had begun to metamorphose at day 93. The MANOVA tested the hypothesis that slopes of the growth profiles were similar (Simms and Burdick 1988), and the series of ANOVAs tested the hypothesis that body sizes were similar at each measurement date.

I analyzed metamorphic size (SVL) and metamorphic timing (days since initiation of experiment) in a 2 x 2 full-factorial MANOVA and ANOVA that examined the effect of food level and temperature on the vector consisting of metamorphic size and metamorphic timing (MANOVA) and univariate analyses on each variable (ANOVA). Data on metamorphic timing were inverse-transformed (Alford and Harris 1988) and data on metamorphic size were $\log_{10}$-transformed (Sokal and Rohlf 1995).

I conducted all analyses in SPSS version 13. Wilks' $\lambda$ was selected as the multivariate test statistic and type III sums-of-squares were used to generate univariate mean squares. Significance was assigned using $\alpha = 0.05$.

## Results

Larvae in different treatments were not different in size at the initiation of the experiment (Table 1, Fig. 1). As predicated by the experiment, larvae fed fewer worms grew more slowly (Fig. 1). Growth profiles began diverging as soon as food treatments were initiated and the slope of the growth profiles continued to be different throughout the experiment (Fig. 1). While food treatment caused larval sizes to be significantly different by day 82, temperature did not result in significant effects on larval body size (Table 1). This result suggests that Blue Ridge Two-lined

Table 1. Summary of 2-way analyses on log-transformed larval size prior to initiation of metamorphosis (i.e., day 93). Multivariate analysis df = 4,30 and univariate df = 1,33 analyses. Values for analyses are *F*-statistics. *P*-values are in parentheses. For the univariate analyses, error mean squares are 0.003 (day 0), 0.002 (day 49), 0.002 (day 68), and 0.002 (day 82). An asterisk (*) indicates significant *F*-tests ($P < 0.05$).

| | | ANOVA | | | |
|---|---|---|---|---|---|
| Source | MANOVA | Day 0 | Day 49 | Day 68 | Day 82 |
| Temperature | 2.45 (0.068) | 0.01 (0.924) | 0.50 (0.485) | 1.40 (0.246) | 0.20 (0.661) |
| Food | 9.33 (<0.001)* | 0.02 (0.892) | 1.66 (0.207) | 3.22 (0.082) | 7.34 (0.011)* |
| Temperature x Food | 0.84 (0.511) | 0.10 (0.753) | 0.01 (0.920) | 0.06 (0.809) | 0.41 (0.527) |

Salamander larval growth is more affected by variation in food levels than by temperature variation.

Larval Blue Ridge Two-lined Salamanders grown at the lower temperature metamorphosed later (Table 2, Fig. 2). The first metamorph (day 93) was from

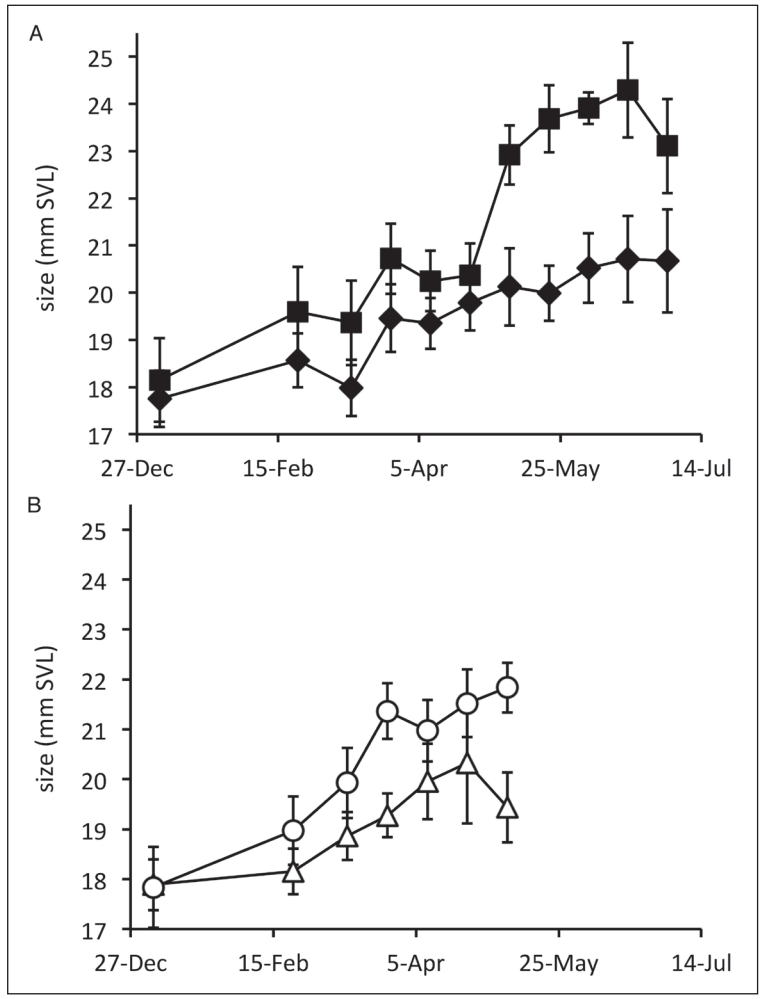


Figure 1. Larval growth profiles of Blue Ridge Two-lined Salamander in (A) cold treatment and (B) warm treatments. Each point is mean ± 1 SD. Lines terminate at last weighing prior to initiation of metamorphosis. Squares and circles are high-food treatments and diamonds and triangles are low-food treaments.

Table 2. Summary of multivariate and univariate analyses on log-transformed metamorphic size and inverse-transformed days to metamorphosis in Blue Ridge Two-lined Salamander. For the analysis of responses by Blue Ridge Two-lined Salamander, df = 2,32 for the multivariate analysis and df = 1,33 for the univariate analyses. Value for univariate analyses are *F*-statistics. *P*-values are in parentheses. Error mean squares are 0.001 for metamorphic mass and 9.91 x $10^{-7}$ for days to metamorphosis. An asterisk (*) indicates significant *F*-tests ($P < 0.05$).

| | Multivariate statistics | | Univariate statistics | |
|---|---|---|---|---|
| Source | Wilks' λ | *F* | Metamorphic mass | Days to metamorphosis |
| Temperature | 0.24 | 49.70 (<0.001)* | 6.20 (0.018)* | 66.15 (<0.001)* |
| Food | 0.67 | 7.91 (0.002)* | 16.18 (<0.001)* | 3.49 (0.071) |
| Temperature x Food | 0.99 | 0.18 (0.840) | 0.37 (0.548) | 0.08 (0.782) |

the high-temperature–high-food treatment, and all high-temperature larvae had metamorphosed by day 138. Only 1 larva from the low-temperature treatment metamorphosed before day 138 (at day 109), and the rest metamorphosed by day 254. Larvae grown at low temperature delayed metamorphosis, they grew for a longer period of time and thus attained larger metamorphic size (7.2%) than larvae at high temperature (Table 2, Fig. 2). In contrast, the food treatment (i.e., growth variation) only strongly affected size at metamorphosis (Table 2, Fig. 2) but had a relatively weak, and non-significant ($P$ = 0.071) effect on timing of metamorphosis. Larvae in the high-food treatments were 12.6% larger at the time of metamorphosis compared to those in the low-food treatments (Fig. 2). The interaction term was non-significant for both responses (Table 2).

## Discussion

The Plethodontidae is, by far, the most diverse family of salamanders, and this family exhibits a wide variety of life cycles: metamorphosis, paedomorphosis, and direct development (Dunn 1926, Wake 1966). The diversity of life histories in the plethodontids and, in particular, the Spelerpini, has been influenced by shifts in developmental timing (Bonett 2016, Bonett et al. 2014, Ryan and Bruce 1998). Indeed, Bonnet (2016) has suggested that species diversity in *Eurycea* is a consequence of life-history diversity, especially in the evolution between paedomorphic and metamorphic life cycles. Phylogenetic evidence suggests that ancestral plethodontids were direct-developers and that subsequent shifts to a

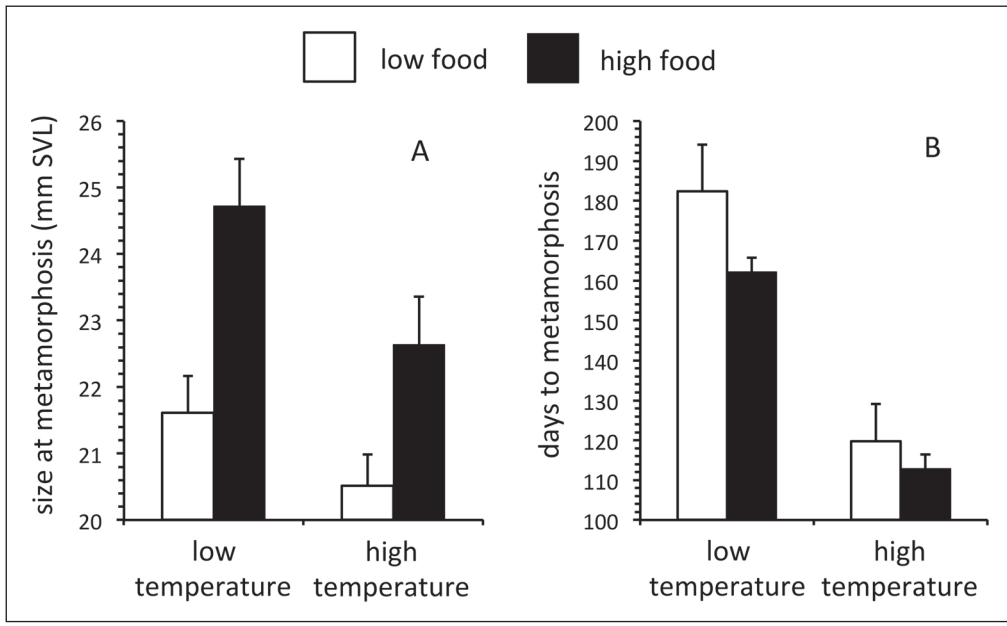


Figure 2. Summary of metamorphic responses of Blue Ridge Two-lined Salamander to temperature and food treatments. (A) Size and metamorphosis in mm SVL. (B) Days from initation of experiment to metamorphosis. Bars are means ± 1 SD. Statistical analysis is summarized in Table 1.

metamorphic life cycle were cases of metamorphic deceleration (Bonnet 2016, Bonnet et al. 2014). Thus, understanding the proximate causes of shifts in metamorphic timing in the Spelerpini seem important in addressing the fundamental question of why this family is so diverse when compared to the rest of salamander families (Beachy et al. 2017).

The effect of temperature is clearly important in affecting metamorphic timing in amphibians. Previous work has shown that growing larval amphibians at low temperature reliably slows development and delays metamorphosis (Beachy 1995, Clarkson and Beachy 2015, Hayes et al. 1993, Hickerson et al. 2005, Leips and Travis 1994, Uhlenhuth 1919) and this was the case in my study. There is a clear connection between these lab studies and natural variation in larval life-histories: cold habitats support larvae that metamorphose later (and often larger) than warmer habitats (Berven and Gill 1983, Smith-Gill and Berven 1979, Voss 1993). In some cases, low temperature appears to be the proximate cause in inducing paedomorphosis (Eagleson 1976).

Although the effect of temperature on amphibian development is clear, the relationship of growth to metamorphic timing is still not well understood. Studies that directly manipulate larval growth by using food treatments are most informative when individuals are isolated from one another, thus avoiding confounding effects of interacting larvae (Alford and Harris 1988). Surprisingly, there are still only ~30 studies across all species in which individual larvae were fed different amounts of food, and metamorphic timing was analyzed (Beachy et al. 2017). It is always desirable to begin growth experiments with embryos and hatchlings, but this is not always possible, e.g., not all species have egg clutches that are easy to find. In any case, and in contrast to predictions of fixed-rate models (e.g., Travis 1984), these studies show that larvae fed different amounts of food metamorphose at different dates, even when the variation in feeding is imposed relatively late in development, i.e., as much as 50–67% of the days needed to complete larval development (Alford and Harris 1988, Beachy et al. 1999, Beachy 2001). Although it is conceivable that beginning this experiment with larvae that are 7 months old (i.e., at 47–58% of the larval period; Bruce 1982, 1988; Voss 1993) limits the interpretation of these results, every experiment (except for those on larval plethodontids) that shifted food availability within this period resulted in variation in metamorphic timing (Beachy et al. 2017).

In studies where growth was directly manipulated using individual larvae, the result of food treatment was generally that larvae grown rapidly metamorphosed earlier (e.g., Hensley 1993, Leips and Travis 1994, Vaissi and Sharifi 2016), although there are cases in the frog genus *Spea* where rapidly growing larvae metamorphose later than larvae that are growing slowly (Denver et al. 1998, Newman 1994). In all lab studies (exclusive of those on plethodontid larvae) where food treatments were imposed, timing of metamorphosis was affected (e.g., Alford and Harris 1988, Beachy 2001, Hensley 1993, Leips and Travis 1994). These studies include insects, crustaceans, frogs, and salamanders (Beachy et al. 2017), and the effects of growth-rate variation were increases or

decreases in mean metamorphic timing that varied from 13% to 66%. In contrast, even if the *P*-value (0.071; Table 2) for the effect of food on metamorphic timing on metamorphosis in Blue Ridge Two-lined Salamander were considered significant, the differences between mean metamorphic timing were only 5.48% (at low temperature) and 2.19% (at high temperature). In the 4 studies that manipulated food levels in larval plethodontids—Beachy (1995), Hickerson et al. (2005), O'Laughlin and Harris (2000), and this study)—changes in mean metamorphic timing varied from <0.1% to 5.48% and were not statistically significant.

It is remarkable that growth-induced plasticity in metamorphic timing is weak (or non-existent) in the plethodontid salamanders, given that it is such a strong determinant of variation in metamorphic timing in other groups of amphibians. It is tenable that the phenotypic plasticity so often inferred to have adaptive value in high-productivity and ephemeral habitats may entail costs that are not economical in the permanent, low-productivity larval habitats experienced by most plethodontid larvae (Beachy et al. 2017). Perhaps this loss of plasticity is important in the evolution of both direct-development and paedomorphosis in the Plethodontidae (Beachy et al. 2017). In contrast, if the ancestral life-cycle of plethodontids is a direct-developing one (Bonett et. 2014), then this lack of plasticity in metamorphic timing is likely a developmental constraint inherited from direct-developing ancestors where plasticity in metamorphosis was disadvantageous.

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