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Experimental Analysis of Allocation during Larval Development in Ambystomatid Salamanders

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Abstract

We examined how variation in food availability and temperature influences patterns of energy allocation to growth, storage, metamorphosis, and reproduction in larval *Ambystoma maculatum* and *A. tigrinum*. In each species, an allocation vector consisting of metamorphic size, duration of larval period, fat body mass, and gonad mass was influenced by both food and temperature. Larvae grew rapidly at high food levels and high temperature, and delayed metamorphosis when grown at low temperature. High food levels resulted in larger fat bodies and decreased time to metamorphosis in *A. tigrinum*, and larger gonads in both species. The allocation vector collectively representing growth rate, storage, reproduction and metamorphosis differed between species: in *A. maculatum*, only the correlation between metamorphic size and fat body mass was significant, whereas all four variables were influenced in *A. tigrinum*. An interaction of food and temperature on the allocation vector was only detected for *A. tigrinum*. Our results indicate that interaction among allocation variables is stronger in some species than others and that these associations might be important in understanding life history variation between these species.

Keywords

Ambystoma; Food; Growth; Metamorphosis; Reproduction; Storage; Temperature

Temperature and food availability have important effects on metamorphic timing in amphibians. Low temperature has the effect of slowing development and delaying metamorphosis (Uhlenhuth 1919; Hayes et al. 1993; Beachy 1995; Hickerson et al. 2005). In almost all experimental studies of growth, food availability influences timing of metamorphosis (e.g., Alford and Harris 1988; Hensley 1993; Leips and Travis 1994; Beachy 2001). However, the relationship between two of these variables—temperature and growth—is complex. In some cases, growth is slowed less than development, with the result that larvae at low temperature can metamorphose later and at larger size (Sexton and Bizer 1978; Beachy 1995); in other cases, larvae metamorphose later at smaller sizes (Hickerson et al. 2005). Resolution to this complexity in metamorphic responses can be obtained by considering energy allocation to other important life history variables (e.g., storage and reproduction; Dunham et al. 1989; Bernardo 1994; Bernardo and Reagan-Wallin 2002; Day

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and Rowe 2002). For example, geographic variation in maturation age in the salamander *Desmognathus ocoee* is, in part, explained by population differences in responses to increases in prey availability (Bernardo 1994).

We explored how the factors of temperature and food availability interact to influence allocation during larval development in two species of *Ambystoma* that differ in life history. Relative to other ambystomatids, *A. maculatum* has slow larval growth and small metamorphic size and is obligately metamorphic throughout its range (Wilbur and Collins 1973; Shoop 1974; Phillips 1992). In contrast, *A. tigrinum* has rapid larval growth and larger metamorphic size, and members of this species complex often express facultative paedomorphosis (Rose and Armentrout 1976; Sexton and Bizer 1978; Collins et al. 1980).

Laboratory growth experiments have aided the understanding of 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). We raised larvae of each species in the laboratory under simulated ecological conditions wherein we manipulated food and temperature and assayed the allocation variables fat body mass, gonad mass, metamorphic mass, and larval period. We hypothesized that larval salamanders that experience different larval environments will have different patterns of allocation and that these differences are associated with species differences in age and size at metamorphosis. For example, we hypothesized that because *A. tigrinum* grows more rapidly than *A. maculatum* that interactions among allocation variables would be stronger in *A. tigrinum*.

Materials and Methods

Our experimental design involved 72 larval *A. maculatum* (18 per treatment) and 96 larval *A. tigrinum* (24 per treatment). We obtained embryos of both species from Charles D. Sullivan, Co. (Nashville, Tennessee). The embryos were wild harvested from a single woodland vernal pool in Wilson Co., Tennessee (35°59'08"N, 86°05'22"W; datum = WGS84) on 21 March 2001. In most years, this pool completely dries by midsummer. Embryos were placed in 20 L of reverse osmosis (RO) water in two 38-L aquaria. Water was replaced every other day. As hatching occurred from 26 to 29 March 2001, each larva was placed in an individual plastic container (28 × 14 × 9 cm, length × width × height) with 950 mL of RO water.

Hatchling larvae were fed freshly hatched brine shrimp (*Artemia* sp.) nauplii. When large enough, larvae were fed California blackworms (*Lubriculus ordinoides*). We hatched 1 teaspoon of brine shrimp eggs (Great Salt Lake Co., Salt Lake City, Utah) in 1 L of full-strength aerated seawater (Instant Ocean, Co.) and drew aliquots of *Artemia* in full plastic 5-mL plastic pipettes. Blackworms were obtained from AquaticFoods.com and were stored in RO water that was changed daily. Brine shrimp were added immediately after water changes. While larvae were fed brine shrimp, water was exchanged daily. Upon switching to blackworms, water was changed either twice weekly or as needed.

We manipulated growth by feeding larvae different amounts of food. Upon hatching, we provided larval salamanders with either one or four aliquots of brine shrimp. As larvae grew,

the number of aliquots of brine shrimp was increased while maintaining a four-fold difference in the number of aliquots of brine shrimp administered between treatments. When larvae were large enough, they were fed blackworms instead of brine shrimp. High food treatment larvae were fed blackworms ad libitum, and low food treatment larvae were fed blackworms twice per week. Low food treatment larvae were fed only as many worms as they could consume in a 24-h period.

Temperature was manipulated by placing larvae in one of two refrigerators (True Co.) equipped with thermostats (Ranco Co.) and glass doors in a room with windows to allow exposure to a natural (Minot, North Dakota) light cycle. Refrigerators were adjacent to each other. Subjects were grown at two temperatures ($\pm 1^\circ\text{C}$): 20 and 16°C. For each temperature, 84 larvae (two temperatures, for 168 larvae in total) in their individual containers (36 *A. maculatum*, 48 *A. tigrinum*) were placed in randomized position in replicate blocks (i.e., each block included containers for each species with one larva from the high food treatment and one larva from the low food treatment). Placement of containers resulted in maximal distance between larvae of 1.7 m in different refrigerators and 0.7 m in the same refrigerator. Placement in separate refrigerators can be confounded with spatial effects. However, the spatial variation in this experiment has not been enough to induce variation in growth rate or metamorphic timing in experiments in our laboratory. Thus, any treatment effects attributable to refrigerator are likely to be caused by our intended temperature treatment.

We assayed allocation to growth, metamorphic development, reproduction, and storage. Growth was assessed by weighing every larva at 30-d intervals. Larvae were removed with an aquarium net from containers, blotted dry on paper toweling while still in the net, and transferred to and weighed in a tared beaker of RO water (± 1 mg). We assayed size at metamorphosis by weighing dry-blotted metamorphosed salamanders. Metamorphosis was determined as complete resorption of gill fimbriae and tail fin. Metamorphic timing was also assayed; date of metamorphosis minus hatching day was used to determine the length of the larval period.

After weighing, metamorphs were euthanized in a 3% solution of MS-222 (Aldrich Sigma), fixed in 10% neutral buffered formalin for 24–48 h, and stored in 70% ethanol. Stored animals were then dissected to assay reproduction and storage. Allocation to reproduction and storage was assayed by removing gonads and fat bodies, respectively, and weighing each of those tissues (± 0.1 mg).

We used a 2×2 full-factorial experimental design that allowed us to statistically analyze the effect of food level and temperature on the vector of allocation variables (using a multivariate analysis of variance) and on each separate allocation variable (using analyses of variance). The multivariate response vector consisted of metamorphic mass, duration of larval period, gonad mass, and fat body mass. Larval period data were inverse transformed (Alford and Harris 1988), and data on metamorphic size, gonad mass, and fat body mass were log transformed (Sokal and Rohlf 1995). All analyses were performed using SPSS (v. 13, SPSS Inc., Chicago, Illinois). Wilks' λ was selected as the multivariate test statistic and Type III sums of squares were used to generate univariate mean squares. In all cases, significance was assigned using $\alpha = 0.05$.

Results

We terminated the experiment after 151 d. All *A. maculatum* and all *A. tigrinum* in the high food treatment had metamorphosed; none of the *A. tigrinum* in the low food treatment had metamorphosed. Average larval periods and sizes of metamorphosed salamanders were within the range of field-collected animals (Wilbur and Collins 1973; Sever and Dineen 1978; Phillips 1992).

Differences in larval mass as a function of treatment were apparent after 30 and 60 d. Larval *A. maculatum* were larger in high food treatments after 60 d ($F_{1,52} = 11.85$, $P = 0.001$) and were larger in high temperature treatments after 30 and 60 d (30 d: $F_{1,52} = 15.90$, $P < 0.001$; 60 d: $F_{1,52} = 9.86$, $P = 0.003$). Larval *A. tigrinum* were larger after 30 and 60 d in high food treatments (30 d: $F_{1,83} = 16.16$, $P < 0.001$; 60 d: $F_{1,83} = 132.01$, $P < 0.001$) and after 60 d in high temperature treatments ($F_{1,83} = 6.45$, $P = 0.01$).

For *A. maculatum*, only the main effects influenced the allocation vector (Table 1). Larvae in the high food treatment had greater metamorphic size and gonad size (Fig. 1). Food treatment did not affect larval period or fat body size. In contrast, the low temperature treatment delayed metamorphosis, but did not affect metamorphic size, gonad size or fat body size (Fig. 1).

For *A. tigrinum*, both the main effects and an interaction between them influenced the allocation vector (Table 1). All larvae in the high food treatment completed metamorphosis, whereas none did in the low food treatment. At the termination of this experiment, larvae in the high food treatment were larger and had larger gonads and fat bodies (Fig. 1). Larvae in the low temperature treatment experienced delayed metamorphosis, increased metamorphic size, and increased fat body mass (Fig. 1). In *A. tigrinum*, only gonad mass failed to show evidence of a food $\times$ temperature interaction. Effects of temperature were more dramatic in high food treatment than in the low food treatment. These effects at high food were complex: larval period, final mass, and fat body size were all reduced at high temperature, whereas gonad mass was increased (Fig. 1). A separate analysis that only included *A. tigrinum* larvae from the high food treatment indicated that larvae raised in the high temperature treatment metamorphosed earlier than those in the low temperature treatment ($F_{1,39} = 64.26$, $P < 0.001$).

Although treatments influenced allocation vectors in both species, the pattern of allocation differed between species. In *A. maculatum*, only metamorphic mass and storage were correlated and only in two of the four treatment groups. In *A. tigrinum*, nearly all responses were correlated (i.e., metamorphic development, larval growth, storage, and reproduction) but these correlations were only seen in the high food treatments (Table 2).

Discussion

Our goals were to determine how (1) temperature variation and food availability affect metamorphic timing and size and (2) differences in larval allocation in response to food and temperature variation contribute to species differences in larval and metamorphic life history parameters. Our results confirm previous studies that show that lower temperature slows

growth and delays metamorphosis (Beachy 1995; Newman 1998; Hickerson et al. 2005). In *A. maculatum*, this delay allowed low temperature larvae to attain metamorphic, fat body, and gonad masses that were similar to those in high temperatures. This outcome indicates that the allocation to growth, storage, and reproduction occurs at a reduced rate at low temperatures. In *A. tigrinum*, the delay in metamorphosis at low temperature was accompanied by larger metamorphic size and greater storage, indicating that allocation to growth and storage is less depressed at low temperatures in *A. tigrinum* compared to *A. maculatum*. This interpretation is supported by the significant food $\times$ temperature interaction in *A. tigrinum* and the absence of this interaction in *A. maculatum*.

Food effects were similar, in part, for both species: Larvae in the high food treatment attained larger size and had larger gonads. Greater food availability resulted in larger fat bodies and accelerated metamorphosis in *A. tigrinum*, but not in *A. maculatum*. The significant interaction between food and temperature in *A. tigrinum* provides evidence *A. tigrinum* are capable of significant increases in allocation to growth and storage because metamorphic and final mass and fat body mass were exceptionally high in high food and low temperature treatments. This finding might be associated with the expression of facultative paedomorphosis in several western North American species of *Ambystoma* that use high productivity ponds or lakes that freeze (or nearly freeze) during winter (e.g., Sprules 1974; Eagleson 1976; Sexton and Bizer 1978; Radi and Beachy 2013).

There were no treatment interactions for *A. maculatum*. However, they did exist for *A. tigrinum* for all allocation variables except gonad mass. For all variables, the influence of high food on accelerating metamorphosis, increasing metamorphic size, and increasing metamorphic mass was more profound at low temperature. This is consistent with the observation that assimilation efficiency is higher at low temperatures in salamanders (Bobka et al. 1981).

Although allocation studies are common, they typically only examine two life history variables: reproduction and growth. In the only other study on allocation in salamanders that included more than these two variables, Bernardo (1994) examined allocation in juvenile *D. ocoee* from different populations in a reciprocal transplant experiment. He reported that juvenile salamanders provided with supplemental food had different allocation response than those raised under ambient food conditions: they grew faster, stored more, and produced larger gonads. This response is consistent with that seen in our larval *Ambystoma*: faster growth, more storage, and larger gonads in both species. This differs from the results of Bernardo (1994) in that the thermal variation in his experiment did not influence growth, storage, or reproductive development. In our larval salamanders, temperature variation induced variation in growth and metamorphic timing in both species and larger metamorphic size and greater storage for *A. tigrinum*. This indicates that species differ in the influence of temperature on allocation, that different stages of the life history (i.e., juvenile vs. larval stage) provide different potential for the influence of temperature variation, or both.

A consideration of storage and reproduction seems to provide clarity to the pattern of association between metamorphic size and timing. For most of the treatment combinations, there were no correlations between metamorphic size and larval period. In *A. tigrinum*

experiencing a high food–low temperature treatment, however, there was a positive correlation between larval period and metamorphic size, and this correlation was reversed at high temperature. When raised at high temperature, the negative correlation between larval period and metamorphic mass was accompanied by a positive relationship between metamorphic mass and fat body mass. Gonad mass was not a strong part of the association. At low temperature, metamorphic mass was positively associated with larval period, gonad mass, and fat body mass.

It can be difficult to detect meaning in patterns of phenotypic correlations among life history variables. In theory, as long as resources are limited, a negative correlation between any two life history variables will exist when allocation to one compromises allocation to the other. The interactions between two variables are also influenced by the relationships of these variables to other allocation variables, however, including unmeasured variables (Bernardo 1994). For both species, correlations among variables were not observed at low temperature–low food conditions. Support for allocation compromises existing among storage, growth, metamorphic development, and reproduction seems to come from *A. maculatum*. Larvae of this species experienced positive storage–body size relationship at low food and high temperature and also at high food–low temperature conditions. This result means that, under these conditions, *A. maculatum* is capable of assimilating enough incoming energy that storage becomes important. This was also true for *A. tigrinum* larvae provided with high food levels.

Day and Rowe (2002) developed an optimization model for metamorphic timing and size. The model output indicated that the apparent discrepancy between the predicted positive association between larval period and metamorphic size proposed by Wilbur and Collins (1973) and negative association observed in experiments and natural populations can be explained by consideration of overhead thresholds. These thresholds imply that other life history features are factored into decisions about energy allocation before and during the metamorphic transition. For example, reproduction and storage might be the life history features that are optimized rather than metamorphic timing. Supplemented with the observation that allocation to growth affects metamorphic timing, the suggestion of Day and Rowe (2002) of overhead thresholds means that the relationship of metamorphic age and size is one of several competing physiological ecological functions to be optimized. Our data support this suggestion. We found that these two species that differ in life history also differ in allocation patterns. It seems tenable that these differences in allocation patterns are related to the rapid larval growth and reproductive development of *A. tigrinum* and that these latter traits might be related to the pattern of facultative paedomorphosis seen in many populations of the *A. tigrinum* species complex.

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Literature Cited

- Alford RA, Harris RN. Effects of larval growth history on anuran metamorphosis. *American Naturalist*. 1988; 131:91–106.
- Beachy CK. Effects of larval growth history on metamorphosis in a stream dwelling salamander (*Desmognathus ochrophaeus*). *Journal of Herpetology*. 1995; 29:375–382.
- Beachy CK. Effects of growth history and thyroxine-induced acceleration on metamorphic timing in the toad *Bufo americanus* (Amphibia, Anura). *Copeia*. 2001; 2001:829–834.
- Beachy CK, Reyes M, Surges T. Effects of developmental and growth history on metamorphosis in the gray treefrog, *Hyla versicolor* (Amphibia, Anura). *Journal of Experimental Zoology*. 1999; 283:522–530. [PubMed: 10194861]
- Bernardo J. Experimental analysis of allocation in two divergent, natural salamander populations. *American Naturalist*. 1994; 143:14–38.
- Bernardo J, Reagan-Wallin NL. Plethodontid salamanders do not conform to “general rules” for ectotherm life histories: Insights from allocation models about why simple models do not make accurate predictions. *Oikos*. 2002; 97:398–414.
- Bobka MS, Jaeger RG, McNaught DC. Temperature dependent assimilation efficiencies of two species of terrestrial salamanders. *Copeia*. 1981; 1981:417–421.
- Collins JP, Mitton JB, Pierce BA. *Ambystoma tigrinum*: A multispecies conglomerate? *Copeia*. 1980; 1980:938–941.
- Day T, Rowe L. Developmental thresholds and the evolution of reaction norms for age and size at life-history transitions. *American Naturalist*. 2002; 159:338–350.
- Dunham AE, Grant BW, Overall KL. Interfaces between biophysical and physiological ecology and the population ecology of terrestrial vertebrate ectotherms. *Physiological Zoology*. 1989; 62:335–355.
- Eagleson GW. A comparison of the life histories and growth patterns of populations of the salamander *Ambystoma gracile* (Baird) from permanent low-altitude and montane lakes. *Canadian Journal of Zoology*. 1976; 54:2098–2111.
- Hayes T, Chan R, Licht P. Interactions of temperature and steroids on larval growth, development, and metamorphosis in a toad (*Bufo boreas*). *Journal of Experimental Zoology*. 1993; 266:206–215. [PubMed: 8515203]
- Hensley FR. Ontogenetic loss of phenotypic plasticity of age at metamorphosis in tadpoles. *Ecology*. 1993; 74:2405–2412.
- Hickerson CM, Barker EL, Beachy CK. Determinants of metamorphic timing in the black-bellied salamander, *Desmognathus quadramaculatus*. *Southeastern Naturalist*. 2005; 4:33–50.
- Leips J, Travis J. Metamorphic responses to changing food levels in two species of hyliid frogs. *Ecology*. 1994; 75:1345–1356.
- Newman RA. Ecological constraints on amphibian metamorphosis: Interactions of temperature and larval density with responses to changing food level. *Oecologia*. 1998; 115:9–16. [PubMed: 28308472]
- Phillips CA. Variation in metamorphosis in spotted salamanders *Ambystoma maculatum* from eastern Missouri. *American Midland Naturalist*. 1992; 128:276–280.
- Radi A, Beachy CK. Natural history notes: *Ambystoma mavortium* (behavior underneath the ice). *Herpetological Review*. 2013; 44:291–292.
- Rose FL, Armentrout D. Adaptive strategies of *Ambystoma tigrinum* Green inhabiting the Llano Estacado of west Texas. *Journal of Animal Ecology*. 1976; 45:713–729.
- Ryan TJ, Semlitsch RD. Growth and the expression of alternative life cycles in the salamander *Ambystoma talpoideum* (Caudata: Ambystomatidae). *Biological Journal of the Linnean Society*. 2003; 80:639–646.
- Sever DM, Dineen CF. Reproductive ecology of the tiger salamander, *Ambystoma tigrinum*, in northern Indiana. *Proceedings of the Indiana Academy of Science*. 1978; 87:189–203.
- Sexton OJ, Bizer JR. Life history patterns of *Ambystoma tigrinum* in montane Colorado. *American Midland Naturalist*. 1978; 99:101–118.

- Shoop CR. Yearly variation in larval survival of *Ambystoma maculatum*. *Ecology*. 1974; 55:440–444.
- Sokal, RR., Rohlf, FJ. *Biometry*. 3. W.H. Freeman; USA: 1995.
- Sprules WG. Environmental factors and the incidence of neoteny in *Ambystoma gracile* (Baird) (Amphibia: Caudata). *Journal of Zoology*. 1974; 52:1545–1552.
- Uhlenhuth E. Relation between metamorphosis and other developmental phenomena in amphibians. *Journal of General Physiology*. 1919; 1:525–544. [PubMed: 19871768]
- Wilbur HM, Collins JP. Ecological aspects of amphibian metamorphosis. *Science*. 1973; 182:1305–1314. [PubMed: 17733097]

Fig. 1.

Average effects of food $\times$ temperature treatments on allocation. Squares represent high food treatments, circles represent low food treatments. Open symbols are for *Ambystoma tigrinum*; closed symbols are for *A. maculatum*. For low food *A. tigrinum*, final mass is mass at termination of experiment; for high food *A. tigrinum* and all *A. maculatum*, final mass is mass at metamorphosis. Vertical lines next to response means indicate similar values using a posteriori contrasts. Only within-species comparisons were performed. Lines connect response means to emphasize the influence of temperature on dependent variables.

Table 1

Summary of multivariate and univariate analyses on larval allocation variable in *Ambystoma maculatum* and *A. tigrinum* salamanders.^a

Source	Multivariate statistics		Univariate statistics			
	Wilks' λ	F	Metamorphic mass	Larval period	Gonad mass	Fat body mass
<i>A. maculatum</i>						
Food	0.17	64.2 (<0.001)	255.8 (<0.001)	2.6 (0.11)	4.6 (0.04)	3.5 (0.13)
Temperature	0.30	31.7 (<0.001)	0.9 (0.36)	122.3 (<0.001)	0.3 (0.59)	1.0 (0.29)
Food $\times$ temperature	0.92	1.2 (0.32)	3.3 (0.07)	0.2 (0.63)	2.2 (0.15)	0.8 (0.34)
<i>A. tigrinum</i>						
Food	0.11	216.6 (<0.001)	469.9 (<0.001)	__ <i>b</i>	18.4 (<0.001)	527.7 (<0.001)
Temperature	0.62	15.9 (<0.001)	17.6 (<0.001)	__ <i>b</i>	0.4 (0.52)	47.4 (<0.001)
Food $\times$ temperature	0.51	25.5 (<0.001)	61.2 (<0.001)	__ <i>b</i>	<0.1 (0.88)	57.3 (<0.001)

^aFor the analysis of responses by *A. maculatum*, df = 4, 53 for the multivariate analysis and df = 1, 56 for the univariate analyses. For the analysis of responses by *A. tigrinum*, df = 3, 79 for the multivariate analysis and df = 1, 81 for the univariate analyses. Values for univariate analyses are *F*-statistics, with those in bold indicating statistical significance (*P*-values in parentheses).

^bLarval *A. tigrinum* in low food treatment did not metamorphose by the end of the experiment; thus, this variable was not included in the analysis. All *A. tigrinum* in high food treatments metamorphosed; metamorphosis was delayed in low temperature treatments (see text).

Table 2

Pearson correlation coefficients between metamorphic (or final^d) mass and other allocation variables in *Ambystoma maculatum* and *A. tigrinum* salamanders.

Species	Temperature	Food level	df	Larval period ^b	Gonad mass	Fat body mass
<i>A. maculatum</i>	Low	Low	17	-0.07	0.23	0.36
	Low	High	16	-0.13	-0.26	0.72 ^{**c}
	High	Low	17	-0.25	0.43	0.69 ^{**}
<i>A. tigrinum</i>	High	High	14	0.01	0.01	0.46
	Low	Low	21	— ^c	0.24	-0.08
	Low	High	22	-0.76 ^{**}	0.60 ^{**}	0.74 ^{**}
	High	Low	23	— ^c	-0.30	0.37
	High	High	19	0.78 ^{***}	-0.08	0.55 [*]

^a *A. tigrinum* larvae maintained on low food did not complete metamorphosis during the experiment.

^b Larval period values were inverse transformed, thus negative correlation coefficients indicate a positive relationship between metamorphic mass and untransformed larval period.

^c Significant values are in bold.

* $P < 0.05$;

** $P < 0.01$;

*** $P < 0.001$.