

## Differences in Larval Allometry among Three Ambystomatid Salamanders

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**ABSTRACT.**—Ecological and evolutionary processes commonly result in morphological variation among larval amphibians. Variation in head shape plays a critical role in both food capture and predation risk in gape-limited salamanders, yet in situ studies of head shape variability are rare outside of cannibal morph assessments. We examined allometry differences in larval head width (HW) and snout–vent length (SVL) among three sympatric species of ambystomatid salamanders from 166 ponds in Missouri, USA: Ringed Salamander (*Ambystoma annulatum*), Marbled Salamander (*A. opacum*), and Spotted Salamander (*A. maculatum*). We also tested whether several abiotic and biotic factors would predict HW after accounting for SVL. We found that larval HW and SVL were strongly correlated for all species but that the strength of this relationship varied among species. For early-stage larvae, Marbled Salamanders showed isometric scaling relationships, whereas both Spotted Salamanders and Ringed Salamanders were allometric. For late-stage larvae, all three species showed allometric patterns. At a small SVL, HW of small Ringed Salamanders was greater than the other species. As larvae increased in SVL, Marbled Salamander HW increased most rapidly and eventually exceeded both Ringed Salamanders and Spotted Salamanders of a similar size. We also found that both abiotic and biotic factors predicted significant differences in HW corrected for SVL among species, including predator density, competitor density, and hydroperiod. Overall, variability in scaling relationships may provide ecological advantages to each species at different points in ontogeny and different biotic and abiotic factors may induce such variation in asymmetric ways among species.

Pond-breeding amphibians show a high degree of morphological variation that can be manifested as continuous traits, such as altered larval tail shapes in response to graded predation threats (Van Buskirk and Schmidt, 2000; Relyea, 2001), or discrete traits, as is the case for trophic polymorphisms or facultative paedomorphosis (Whiteman, 1994; Denoel et al., 2005; Pfennig et al., 2007). Such trait variation in larval amphibians occurs in response to numerous mechanisms, including phenotypic plasticity, natural selection, and micro-evolutionary divergence (Van Buskirk, 2009). The consequences of morphological variation are important for both ecological and evolutionary processes, as such changes can enhance individual survival rates, as well as be the basis for evolution and speciation (Pfennig et al., 2010).

Variation in morphological traits often scales with overall body size; however, the degree of such correspondence can range from nearly perfect covariance of a trait with body size (i.e., isometry) to highly uncorrelated, where specific morphological traits change more or less rapidly with increasing body size (i.e., allometry). Among larval salamanders, scaling relationships of head shape with body size have been investigated in three contexts. First, variation in head shape while correcting for body size has been examined in association with cannibalism, either with species that exhibit known cannibalistic morphs (e.g., Tiger Salamander [*Ambystoma tigrinum*]; Pierce et al., 1983, Sheen and Whiteman, 1998) or in tests to determine whether a cannibal morph exists (Nyman et al., 1993, Walls et al., 1993a,b; Jefferson et al., 2014). Second, variation in head shape has been investigated in experimental studies of the effects of several selective pressures, including larval diet and predation risk (Schmidt and Van Buskirk, 2005; Schmidt et al., 2006; Urban, 2008, 2010; Van Buskirk, 2011; Shaffery and Relyea, 2015). The results of such experiments

have shown that exposure to predation threats can induce larger head widths, as can certain prey types. Finally, morphological variation (including head shape) from in situ studies exhibits a high degree of variability among species in response to numerous environmental factors, including canopy cover and the amount of vegetation in ponds (Van Buskirk, 2009). These responses varied from positive, negative, or neutral, in addition to exhibiting asymmetric strengths in those relationships to environmental constraints.

For salamander species that do not exhibit a cannibalistic morphology, less is known about head shape and body size scaling relationships (but see Harris, 1989; Hasumi and Iwasawa, 1990; Nishihara, 1996), or the processes that cause such variation, if present. Because larval salamanders are gape-limited predators, individuals are predicted to gain foraging and predatory advantages over competitors and prey, respectively, if head width scales differently with body size within or among species. Also, larger head size for a given body length may reduce predation risk by other predators, including con- and heterospecifics (Urban, 2008; Van Buskirk, 2011). Other factors, such as greater canopy cover, also predict increased head widths, although the exact mechanism is not clear (Van Buskirk, 2011). Additional comparisons of scaling relationships among different species of larval salamanders, as well as investigations of the factors that would predict such interspecific differences, would therefore improve our understanding of such morphological variation.

We tested whether three species of ambystomatid salamanders showed different scaling relationships of head width (HW) to snout–vent length (SVL). We also examined different factors that we expected would influence scaling relationships: predator density, larval ambystomatid density, hydroperiod, pond vegetation, and canopy closure. Based on previous work, we predicted that predatory salamander densities, larval invertebrate predator densities, and canopy cover would induce larger heads among larval salamanders (Urban, 2008; Van Buskirk,

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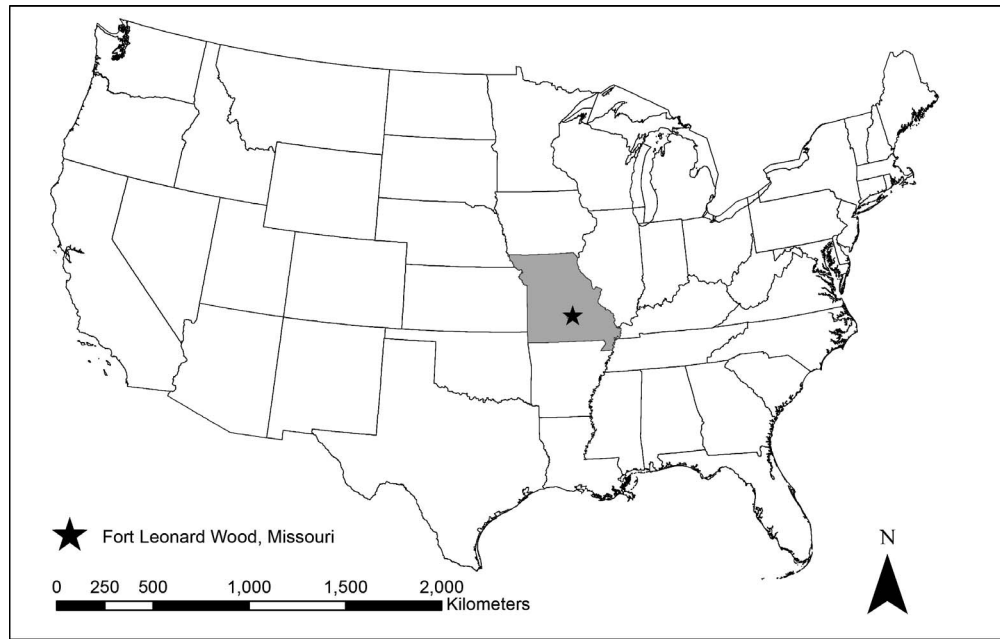


FIG. 1. Location of Fort Leonard Wood, Missouri, USA.

2011; Shaffery and Relyea, 2015). We also expected strong variability among species (Van Buskirk, 2009), although the exact direction of such asymmetries was not predicted, as few studies have examined morphological variation in our focal species.

#### MATERIALS AND METHODS

*Study Species.*—Ringed Salamanders (*Ambystoma annulatum*), Marbled Salamanders (*A. opacum*), and Spotted Salamanders (*A. maculatum*) co-occur in the Ozark and Ouachita mountains of Missouri, Arkansas, and Oklahoma (Petranka, 1998). Both Ringed Salamanders and Marbled Salamanders are fall-breeding species, whereas Spotted Salamanders are a spring-breeding species. Adults of all three species migrate to ponds following rainfall events coupled with cooler (Ringed Salamanders and Marbled Salamanders) and warmer (Spotted Salamanders) temperatures. Larvae for the fall breeders overwinter in ponds and undergo metamorphosis in the late spring and early summer (Hocking et al., 2008; Semlitsch et al., 2014). The larval period length for Spotted Salamanders is approximately 2–6 mo, with metamorphosis occurring primarily in mid-to-late summer (Semlitsch and Anderson, 2016). Larval Ringed Salamanders and Marbled Salamanders both are considered cannibalistic (Nyman et al., 1993; Walls and Blaustein, 1995), although neither exhibit true cannibal morphology (e.g., vomerine teeth and broader head); larval Spotted Salamanders also are likely to be cannibalistic, although this has not been investigated. Interspecific predation also occurs among larvae of all three species if size differences among individuals are great enough (Urban, 2007; Anderson and Semlitsch, 2014; Anderson et al., 2016).

*Data Collection.*—Our field data come from a large landscape study of spatiotemporal dynamics of pond communities at Fort Leonard Wood, Missouri, USA (see Peterman et al., 2014; Anderson et al., 2015; Ousterhout et al., 2015; Peterman et al., 2015; Fig. 1). To briefly summarize our capture methods, from 2012 to 2014 we surveyed approximately 200 ponds by using dipnets and minnow traps repeatedly over a 3-d period,

occurring approximately two-thirds of the way through each salamander species' larval period (approximately March and late May/early June for fall and spring breeders, respectively; hereafter, early-stage larval sampling period). We also surveyed ponds occupied by salamanders with dipnets on a single visit as each species neared metamorphosis (approximately late April/early May and late June/early July for fall and spring breeders, respectively; hereafter, late-stage larval sampling period). We attempted to capture and photograph 20 larvae per species-pond-year when possible. We dorsally photographed larvae in a white metal dissecting tray that was filled with water and a ruler. All larvae were returned unharmed to their pond after photographing. During these surveys, we also recorded the total number of predators that we expected would influence body size. Specifically, we summed the total number of individuals of invertebrate predators captured per pond, including Aeshnidae, larval Dytiscidae, crayfish, and Belostomatidae (primarily *Belostoma* spp. and a few *Lethocerus* spp.), all of which have shown some ability to prey upon larval amphibians (Brodie and Formanowicz, 1983; Van Buskirk, 1988; Gamradt and Kats, 1996).

We did not measure or differentiate predator size classes or species, which we recognize could influence their predatory abilities. Given a goal of the study was to sample ~200 ponds within a short temporal period, collecting and measuring larval invertebrates was not feasible. However, in the springtime most aeshnids are large overwintering instars that are capable of preying upon most size classes of larval salamanders (Wisinger, 1988). Aeshnids, belostomatids, and dytiscids also are generally considered gape-unconstrained predators because of their foraging morphology and tactics, such as stiff prementum, piercing mouthparts, or necrotizing venom (Formanowicz, 1982; Brodie and Formanowicz, 1983; Urban, 2008). We also recorded the number of adult Central Newts (*Notophthalmus viridescens louisianensis*), predators of early ontogenetic stages of all three species and competitors with late-stage larvae. We calculated densities of each group separately as catch per unit effort (CPUE); i.e., the number of individuals captured divided by total sampling effort (combined number of dips and traps).

TABLE 1. Parameter estimates and 95% profile confidence intervals (CI) from the log-log linear analysis of covariance mixed effects model predicting log (HW). Fixed effects included species, survey period, log(SVL), and random effects included SVL, pond and year as random effects. Log(SVL) was centered before analysis.

Salamander species	Early stage				Late stage			
	Intercept	CI	Slope	CI	Intercept	CI	Slope	CI
Ringed	2.006	1.944,2.031	0.704	0.703,0.707	1.974	1.973,1.998	0.644	0.643,0.646
Spotted	1.869	1.845,1.893	0.864	0.863,0.865	1.848	1.848,1.851	0.800	0.799,0.802
Marbled	1.933	1.908,1.957	1.013	1.012,1.015	1.955	1.955,1.957	0.724	0.724,0.729

We measured HW and SVL of all larvae by using ImageJ (Rasband, 1997). We defined SVL as the distance from the snout to the distal juncture of the back legs to the tail and HW as the widest point across the head perpendicular to the body axis. We did not measure larvae that were covered in a way that impeded visibility, or if the body was contorted such that we felt the measurements would not be accurate. We did not measure other variables associated with morphology that are typical of studies investigating potential cannibal morphs (e.g., enlarged vomerine teeth), as we did not collect and euthanize larvae because of the concurrent population surveys of our sites.

**Analysis.**—We assessed differences among species in their relationship of HW to SVL by using a linear analysis of covariance mixed effects model, with species, sampling period, and year as categorical fixed effects. We included pond and SVL as random effects to account for heterogeneity in intercepts and slopes. We included sampling period as a fixed effect, as these were discrete time points within each species' life history and would allow us to assess whether scaling changed through time. Following other studies (Nishihara, 1996; Sheen and Whiteman, 1998; Jefferson et al., 2014), we natural log transformed both HW and SVL, which is equivalent to testing whether the data exhibit a power law relationship ( $y \sim a x^b$ ); when the fit produces a significant linear relationship, the slope term is analogous to the exponent.

We then examined different biotic and abiotic factors that were expected to influence variation in body size allometry within each species. We extracted the residuals of a log-log regression performed on all species, but only for the early-stage larval data (from dipnets and traps) because we did not record predator numbers during the late-stage larval sampling period. We initially compared several linear and nonlinear models to ensure all effects of SVL were removed from influencing HW, and found that  $\log(\text{HW}) \sim \sqrt{\log(\text{SVL})} + \log(\text{SVL}) + \log(\text{SVL}^2)$ , provided the best fit based on Akaike's Information Criterion (Burnham and Anderson, 2002). We used the residuals from this model as the response variable, as this would test for the effects of biotic and abiotic factors on HW independent of SVL (e.g., Urban, 2010; hereafter, relative HW). The predictor variables for each model were larval salamander species, larval salamander CPUE, larval Aeshnidae CPUE, larval dysticid CPUE, Belostomatidae CPUE (including both *Belostoma* and *Lethocerus*), adult Central Newt CPUE, hydroperiod (four categories: ephemeral, summer drying, semipermanent, and permanent), percentage of canopy cover (measured with a spherical densiometer at four points facing all four cardinal directions per pond in summer 2012), and the percentage of pond surface area covered by vegetation (visually estimated during summer 2012). We then included all interaction terms of salamander species with each covariate to test whether each species differed in their response. We again included year as fixed effect and pond as a random effect.

We used the predator density estimates for each group from the March sampling only, as this number best represented the predator density experienced by all three focal species. Larval salamander density from this time point represented the presumed effect of competition between Ringed Salamanders and Marbled Salamanders, but signified the predatory threat from these species on Spotted Salamanders as breeding in this latter species occurred simultaneous with sampling. We centered and scaled all continuous covariates before analyses (i.e., subtracted the mean and divided by the standard deviation), and assessed the significance of each term using the Anova function in the 'car' package (Fox and Weisberg, 2011). We found no evidence of collinearity, as all correlations among predictor variables were relatively low (all  $r < 0.2$ ). All models were constructed using the 'lme4' package in R (Bates et al., 2015; R Development Core Team, 2015).

## RESULTS

We captured and measured 14,990 larvae in total over 3 yr in 166 ponds (6,177 Ringed Salamanders; 6,619 Spotted Salamanders; and 2,194 Marbled Salamanders). Of these larvae, we were able to measure 10,982 larvae for both SVL and HW (3,601 Ringed Salamanders; 6,157 Spotted Salamanders; and 1,224 Marbled Salamanders). Our average sample sizes per pond per year were 24 Ringed Salamanders, 25 Spotted Salamanders, and 13 Marbled Salamanders.

In the early larval period, Marbled Salamanders exhibited nearly perfect isometry of HW to SVL, whereas both Ringed Salamanders and Spotted Salamanders exhibited allometric scaling (Table 1; Fig. 2). In the late larval period, all three species showed allometric scaling (Table 1; Fig. 2). For the log-linear model,  $\log(\text{HW})$  was predicted by a significant three-way interaction among sampling period, species, and  $\log(\text{SVL})$ . For both the early- and late-stage larvae, all species had significantly different intercepts (Table 1; Fig. 2). In both survey periods, Marbled Salamanders had a significantly steeper slope than Ringed Salamanders and Spotted Salamanders (Table 1), which were not different from each other. After centering  $\log(\text{SVL})$ , Marbled Salamanders and Ringed Salamanders had nearly identical intercepts that were both larger than Spotted Salamanders (Fig. 2).

Relative HW was explained by a significant interaction between larval salamander species and larval salamander CPUE ( $\chi^2 = 2$ ,  $df = 2$ ,  $P < 0.001$ ), dysticid CPUE ( $\chi^2 = 2$ ,  $df = 2$ ,  $P = 0.03$ ), and hydroperiod ( $\chi^2 = 2$ ,  $df = 6$ ,  $P < 0.001$ ). Marbled Salamanders showed a significant positive relationship, Ringed Salamanders a negative relationship, and Spotted Salamanders no relationship with larval salamander CPUE (Fig. 3A). For hydroperiod, relative HW was smaller for Ringed Salamanders in more ephemeral ponds compared to the other two species (Fig. 3B). Relative HW for Ringed Salamanders

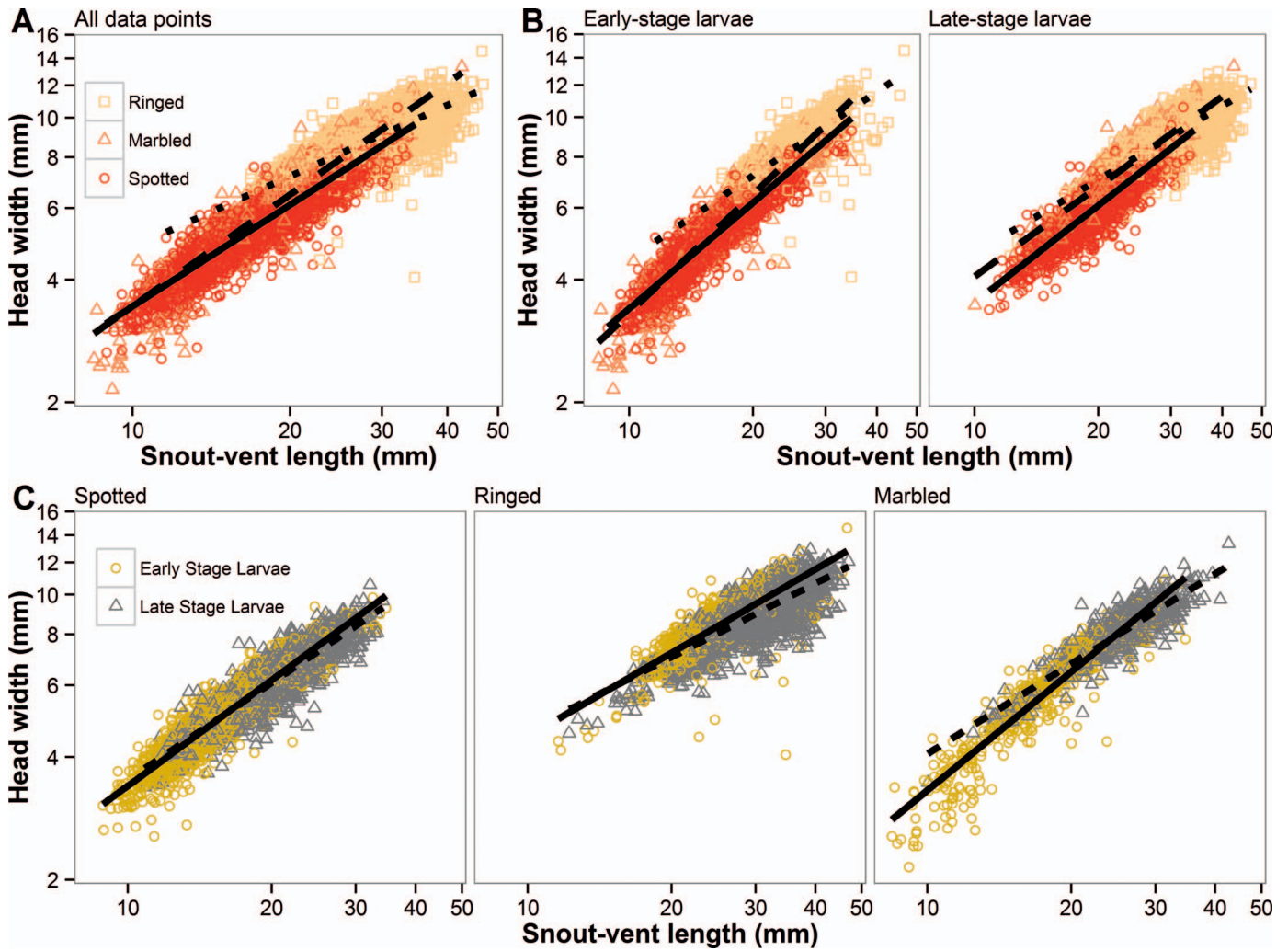


FIG. 2. Relationship of head width and snout-vent length for larval Ringed Salamanders, Marbled Salamanders, and Spotted Salamanders. All panels show the same data, but separated in different ways. (A–B) Dotted line and square symbols = Ringed Salamanders, dashed line and triangle symbols = Marbled Salamanders, and solid line and circle symbols = Spotted Salamanders. (C) Solid line and circle symbols = early-stage larvae and dashed line and triangle symbols = late-stage larvae.

increased in more permanent ponds, whereas relative HW for Spotted Salamanders decreased slightly as hydroperiod increased (Fig. 3B). Relative HW was greatest for Marbled Salamanders in ephemeral and permanent ponds, and decreased in seasonally drying ponds (Fig. 3B). Spotted Salamanders had reduced relative HW at higher dytiscid CPUE, whereas both Marbled Salamanders and Ringed Salamanders showed positive relationships. There were no significant differences among species for relative HW were observed in relation to newt CPUE, percentage of canopy cover, percentage of aquatic vegetation, aeshnid CPUE, crayfish CPUE, or belostomatid CPUE (Fig. 3D–I).

#### DISCUSSION

The three focal salamanders in this study exhibited different scaling relationships between HW and SVL. We also observed species-specific and temporally variable patterns in allometric scaling. Primarily, the strength of scaling changed for Marbled Salamanders from isometric to allometric across the early- to late-stage larval periods, respectively, whereas such shifts were absent or greatly reduced for the other two species. Finally, we identified several abiotic and biotic factors that influenced

relative HW, but the strength and direction of these effects varied among species. Overall, these findings indicate that 1) scaling relationships vary among species, 2) shifts in scaling can occur across ontogeny, and 3) variability in amphibian morphology among species in natural environments corresponds to environmental constraints in different ways. Identification of such patterns, as well as potential causal mechanisms, is critical because such morphological variation can shape both the ecology and evolution of numerous taxa.

In general, at smaller body sizes, Ringed Salamanders had the greatest HW of the three species. As larvae progressed in development, Marbled Salamanders caught up to and slightly surpassed Ringed Salamanders in HW at larger body sizes, because of their isometric pattern compared with allometric patterns in Ringed Salamanders. This was most pronounced using the late-stage larvae dataset, which is interesting given that Marbled Salamanders also showed a significant decline in slope for this period (i.e., increasing allometry among late-stage larvae). Yet, larval Marbled Salamanders still surpassed HW of Ringed Salamanders. Because HW dictates the size of prey that can be captured (Walls et al., 1993b), such shifts in allometric scaling suggest that competitive abilities may change over time.

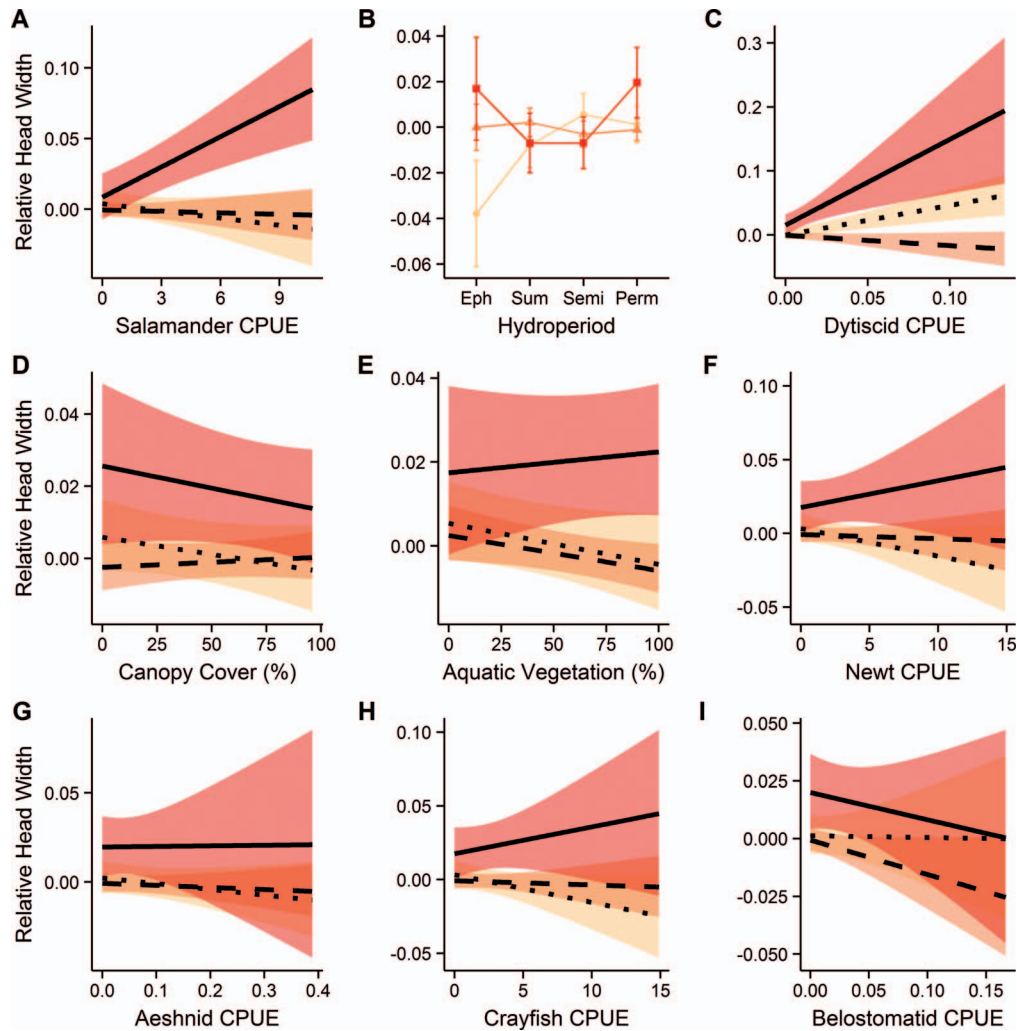


FIG. 3. Predicted relationships of relative HW with abiotic and biotic relationships for early-stage larvae of Ringed Salamanders (dotted line, red shading), Marbled Salamanders (dashed line, orange shading), and Spotted Salamanders (solid line, yellow shading). In (B), symbols represent predicted mean values, where circles = Ringed Salamanders, squares = Marbled Salamanders, and triangles = Spotted Salamanders. X-axis categories in (B) include “eph” = ephemeral, dries multiple times a year; “sum” = summer, dries in the summer every year, “semi” = semi-permanent, dries only occasionally and “perm” = permanent, never dries. Shaded areas and error bars represent 95% confidence intervals.

For example, Ringed Salamanders may have competitive or foraging advantages over Marbled Salamanders early in ontogeny, but this pattern switches among larger larvae later in ontogeny. Previous studies support this observation, as late-stage Marbled Salamanders captured congeneric prey at a faster rate than late-stage Ringed Salamanders of an equivalent size (Anderson et al., 2016). Head width of Ringed Salamanders also may inherently increase more rapidly from hatching to early-stage larvae than the other species, giving them an initial advantage (Pierce et al., 1983); however, HW of larval salamanders at the time of hatching has not been evaluated among these species to verify this hypothesis.

Many studies that examine HW–SVL relationships do so in the context of assessing cannibal morphology in larval salamanders. We did not make detailed measurements of head shape to assess whether other morphological traits varied with SVL among species and therefore could not test for potential cannibal morphs in our focal species. Cannibalism has been observed in both fall-breeding species in this study, and Ringed Salamanders and Marbled Salamanders can both consume Spotted Salamanders (Nyman et al., 1993; Walls and Blaustein, 1995; Anderson et al., 2016), but none of these species have

exhibited morphology similar to cannibalistic trophic polymorphisms in Tiger Salamanders (Sheen and Whiteman, 1998) or Long-toed Salamanders (*Ambystoma macrodactylum columbianum*) (Walls et al., 1993a). We posit that the instances of cannibalism in Ringed Salamanders reported by Nyman et al. (1993) and Jefferson et al. (2014) stem from intraspecific phenology variation that resulted in cohorts hatching in ponds at disparate times in the fall, permitting cannibalism to occur. Such breeding asynchrony would be needed to induce size variation among individuals, as mesocosm experiments indicate high survival in this species despite high densities, and very few sublethal predation attempts (Anderson and Semlitsch, 2016; Ousterhout and Semlitsch, 2016). The relative timing of breeding has not been compared for Marbled Salamanders and Ringed Salamanders, but it may be another mechanism that permits interspecific predation to occur if one species breeds earlier and attains a size advantage. We speculate hydroperiod may play a critical role in this process, because of the differences in egg-laying strategy and hydroperiod preferences that would influence which species’ eggs would hatch first (Petranka, 1998; Peterman et al., 2014).

We identified significant relationships between relative HW with competitor CPUE, predator CPUE (both salamanders and invertebrates), and hydroperiod that varied in direction and strength among the focal species. Such asymmetric patterns among species match the findings of other multispecies investigations (Relyea, 2001; Van Buskirk, 2009; Shaffery and Relyea, 2015), indicating species-specific responses in morphological variation to environmental stimuli is likely a general phenomenon among amphibians. We observed the strongest interspecific variability in response to hydroperiod and salamander CPUE. Under more ephemeral hydroperiod regimes, Ringed Salamanders had a greatly reduced relative HW compared to Marbled Salamanders and Spotted Salamanders, which may be explained by life histories. Marbled Salamanders and Spotted Salamanders each select more ephemeral habitats (Peterman et al., 2014) because of egg-laying strategies (Marbled Salamanders) and shorter larval period requirements (Spotted Salamanders), potentially resulting in altered morphology to enhance foraging in these environments. In contrast, Ringed Salamanders may trade off growth in HW and body size to ensure completion of metamorphosis before pond drying. However, Marbled Salamanders also had the greatest relative HW in permanent ponds; this result could reflect a greater responsive of this species to increased predator densities in more permanent fishless ponds (Schneider and Frost, 1996; Semlitsch et al., 2015) or other features of these environments (e.g., different prey base). Spotted Salamanders did not show substantial variation in relative HW under differing hydroperiod regimes, which corresponds to their life history strategy of metamorphosing quickly and at smaller body sizes compared to the other two species (Petranka, 1998).

Ringed Salamanders showed decreasing relative HW with increasing larval salamander CPUE, whereas Marbled Salamanders showed increased relative HW. This finding suggests that at high competitor densities, Marbled Salamanders may have competitive advantages, assuming greater HW permits greater foraging abilities. Marbled Salamanders have the highest relative foraging rates for a given body size compared to the other two focal species when consuming congeneric prey (Anderson et al., 2016), supporting this hypothesis. At low competitor densities, species are more equivalent in relative HW, reducing the strength of asymmetric interactions. Spotted Salamanders showed no significant relationship with increasing CPUE of fall-breeding salamanders, indicating density-dependent predation or competition from congeners did not result in increased relative HW, similar to the results observed by Urban (2010).

We observed increased relative HW for both Ringed Marbled Salamanders and Spotted Salamanders and Marbled Salamanders, and slightly decreased relative HW for Spotted Salamanders with increasing dytiscid CPUE. The exact mechanism of this result is not known, as larval dytiscids are generally gape-unlimited predators that use venom to subdue prey, resulting in minimal changes to ambystomatid morphology (Formanowicz, 1982; Brodie and Formanowicz, 1983; Urban, 2010). Further work is needed to determine why larvae of fall-breeding species show a greater increase in HW to increasing dytiscid densities.

We observed no relationship with newts or aeshnid dragonflies, an interesting observation given other studies have found that these predators can induce morphological changes in other larval amphibians (Relyea, 2001; Van Buskirk, 2011). Because ambystomatids can grow to exceed the gape limitations of newts rather quickly, their presence may not induce strong

morphological variation. Aeshnids induced varying relative HW in other ambystomatids (Shaffery and Relyea, 2015); therefore, it is unclear why they had no effect in this study. It is possible that our omission of controlling for predator size influenced this result. Other factors that were not measured at our sites could also influence relative HW or other allometric scaling relationships. In particular, diet has been shown to influence head shape in larval salamanders (Walls et al., 1993b; Schmidt et al., 2006), but we did not have data on this aspect of the food web to test this factor.

Herpetologists need to consider whether morphological variation or differences in allometric scaling imparts biologically relevant responses in addition to statistically significant effects. The few in situ studies that investigated morphological variation in amphibians have found that it was consistent with patterns observed in experimental studies designed to isolate mechanisms of interest (e.g., competition or predation; Van Buskirk, 2009). Therefore, the patterns we observed likely represent realistic and biologically relevant responses to biotic and abiotic pressures, although we recognize morphological variation has been less intensively studied in our focal species compared with other amphibian taxa that would permit mechanistic explanations of our observed patterns. As identification of the importance of such pressures can be difficult in situ, increasing the frequency of coupled experimental and field studies (e.g., Urban, 2008; Van Buskirk, 2009, 2011) would further corroborate the drivers of morphological variability or body scaling relationships, and improve our overall understanding of the mechanisms that induce phenotypic variation.

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