

# Effects of Larval Density on Hematological Stress Indices in Salamanders

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**ABSTRACT** In animals with complex life cycles, the quality of juvenile environments is important in shaping the longer-term fitness of individuals. Larval density is a major factor governing quality of larval environments in amphibians, with high densities leading to reduced growth rates, smaller size at metamorphosis, and potentially long-lasting postmetamorphic effects. A little-studied effect of larval density is its impact on physiological stress of postmetamorphic individuals. We used a hematological approach, involving counts of specific white blood cells types (neutrophils and lymphocytes) that covary with corticosterone, to estimate stress levels in recently metamorphosed spotted salamanders (*Ambystoma maculatum*) that were reared in three different larval densities in outdoor mesocosms. In replicated treatments consisting of 12, 25, or 50 larvae, survival was, as expected, lowest and size at metamorphosis smallest in the highest density mesocosms. In addition, surviving salamanders from high-density treatments had significantly higher neutrophil to lymphocyte ratios, indicative of high levels of stress hormones (corticosterone). This trend was not a result of density-related differences in body condition as these did not vary with density. Further, estimated stress levels were similar regardless of whether the salamanders metamorphosed early or late, suggesting that the density effect on stress is long-lasting even once realized density has been reduced through mortality or early metamorphosis. These results may be important in understanding amphibian population dynamics, since research on other vertebrate taxa demonstrates that high hematological stress indicators lead to reduced growth, survival, and increased disease susceptibility in vertebrate animals. *J. Exp. Zool.* 311A, 2009.

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In animal species with complex life cycles, it is important for zoologists to consider how the fitness of any individual can be greatly influenced by its experiences in earlier life stages. Amphibians are a prime example, since many species have aquatic larval stages that eventually metamorphose into morphologically distinct terrestrial forms, and numerous examples show how larval environments can influence the performance of postmetamorphic individuals (e.g. Werner, '86; Semlitsch et al., '88; Scott, '94; Beck and Congdon, 2000; Scott et al., 2007; Gervasi and Fofopoulos, 2008). One of the most important factors that determine the quality of larval environments in amphibians is the density of individuals (e.g. Wilbur, '76; Petranka, '89; Semlitsch and

Reichling, '89; Loman, 2004), with high larval densities invariably leading to a number of detrimental effects, such as reductions in larval growth or size at metamorphosis (Collins, '79; Semlitsch and Caldwell, '82; Travis, '84; Altwegg and Reyer, 2003). Aside from these traditional measures of larval fitness, there is also growing evidence that high larval density and/or food

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limitation also leads to increases in physiological stress of amphibian larvae (Glennemeier and Denver, 2002b; Crespi and Denver, 2005; Hu et al., 2008).

Physiological stress in amphibians, as with all vertebrates, is usually inferred when increases in glucocorticoid hormones are detected (reviewed in Wingfield and Romero, 2001). These hormones (corticosterone in amphibians, birds, and reptiles) are released into the bloodstream when animals perceive harmful stimuli, where they orchestrate a number of physiological changes thought to promote survival. If the stimulus is short-term or "acute" in nature, such as being chased by a predator, a temporary increase in corticosterone occurs, followed by a return to baseline levels within hours after escape (Langkilde and Shine, 2006). Alternatively, "chronic" stressors such as high larval density in amphibians (which lasts for the duration of the larval period and which the larvae cannot necessarily escape from) can lead to chronically elevated baseline levels of glucocorticoid hormones in the larvae (Glennemeier and Denver, 2002b). Chronic stress can be detrimental to animals if occurring over long time periods, with known effects including suppression of immune activity (Martin et al., 2005; Kiank et al., 2006) and even disruption of the aforementioned acute stress response (Hull et al., 2007).

Stress-related increases in corticosterone have an additional effect on circulating leukocyte populations that is less well-known, but which conveniently provides researchers with a way to indirectly assess chronic stress in vertebrates (reviewed in Davis et al., 2008). In all vertebrates, there are five different types of white blood cells: neutrophils, lymphocytes, eosinophils, basophils, and monocytes (Jain, '86), and stress-induced increases in glucocorticoid hormones cause characteristic alterations in the circulating numbers of two of these cell types. Specifically, increases in these hormones invariably lead to increases in the number of neutrophils (or heterophils, the avian and reptilian equivalent) and decreases in lymphocytes in the circulating blood (Gross and Siegel, '83; Bennett, '86; Dhabhar et al., '96). The reason for these alterations in circulating cell numbers is thought to be so that the cells are routed, or "redistributed," to where they would be most needed during stressful events (Dhabhar et al., '96). Specifically, glucocorticoid hormones cause neutrophils, which are phagocytic and attack foreign substances, to be released into the bloodstream from reserve banks in tissues or on

capillary walls, while they cause lymphocytes to emigrate from the blood into tissues and simultaneously be retained from recirculation within the hemopoietic tissue (Dhabhar et al., '95). Other cell types appear to be less affected by stress hormones (Davis et al., 2008). Thus for the researcher, the ratio of the numbers of neutrophils and lymphocytes detected from standard blood smears (the neutrophil-lymphocyte or "N/L" ratio) can be effectively used to infer levels of stress hormones, and in effect to estimate physiological stress (i.e. baseline levels) in animals in a variety of natural or experimental settings (reviewed in Davis et al., 2008). Indeed, recent work by the authors shows this hematological approach is useful for assessing chronic stress in salamanders (Davis and Maerz, 2008a,b).

Unlike other vertebrates, amphibians must also contend with the stress of metamorphosis in the transition from larval to adult form. During this transition, corticosterone is produced and is involved in the reorganization of the amphibian immune system (Rollins-Smith et al., '97). Indeed, levels of this hormone show a general increase during metamorphic climax (Glennemeier and Denver, 2002a; Krain and Denver, 2004). Curiously though, during this time there is no corresponding increase in circulating neutrophil leukocytes as is seen during typical stress responses (Rosenkilde et al., '95; Davis, 2009), perhaps because of the changes occurring in the immune system during the metamorphosis, or perhaps the metamorphosis-related stress differs from the typical stress response in some manner. Regardless of the reason, for the amphibian researcher this result means that neutrophil-lymphocyte ratios of amphibians taken during the metamorphic period would not be unduly influenced by the "stress" of metamorphosis.

This study is aimed at elucidating the relationships between larval density, a known stressor, and physiological stress levels of postmetamorphic amphibians. Specifically, given the known effect of density on the quality of amphibian larval environments and stress of larvae, we questioned if individuals survive this environment, do they then enter the terrestrial world with elevated stress (i.e. after they finish metamorphosis)? Thus, our goal in this study was to determine whether larval density affects "baseline" stress levels of recently metamorphosed spotted salamanders (*Ambystoma maculatum*) as revealed by hematological stress indices (i.e. neutrophil-lymphocyte ratios). We reared salamander larvae at three densities to

create environments of varying quality, which we compared neutrophil-lymphocyte ratios and traditional measures of larval performance across: survival and time to metamorphosis, mass, and body condition.

## MATERIALS AND METHODS

### *Rearing larvae*

Fourteen egg masses of *A. maculatum* were gathered from natural, ephemeral ponds near Athens, GA (33.88° latitude, -83.36° longitude) in February 2006 and housed in separate containers until hatching. Within 3 days after hatching, larvae were haphazardly assigned to one of three groups: three replicate groups of 12 larvae (low-density treatment), three groups of 25 (medium density), and three groups of 50 (high density), for a total of 261 larvae. Nine nonpermeable, plastic mesocosms (i.e. "cattle tanks," with 1,000 L capacity) were submerged halfway in an impounded, permanent pond in the Whitehall Experimental Forest near Athens, GA. The pond was surrounded by mixed hardwood and softwood trees, and contained no fish. The mesocosms were each filled with approximately 650 L of filtered pond water and approximately 2 L of leaf litter was placed in the bottom of each. With this arrangement, the conditions in each mesocosm (i.e. diurnal and monthly water and air temperatures) therefore mimicked those of the pond, except for the absence of aquatic predators (i.e. turtles, bullfrogs, etc.) and other amphibian species. Moreover, mesocosms were not covered so that natural colonization or oviposition by aerial insects would take place and serve as a larval food resource (mesocosms were not supplemented with food during the experiment). This also made it possible for aquatic insect predators to colonize the mesocosms (i.e. dragonfly larvae), though we did not observe any during the experiment.

Each mesocosm was randomly assigned to one of the three treatments (i.e. so that the treatments were interspersed within the mesocosm array) and on March 29, larvae were added. Since each mesocosm contained 650 L of water, the realized larval density for each mesocosm was therefore approximately 0.02 larvae/L, 0.04 larvae/L, and 0.08 larvae/L for the low, medium, and high densities, respectively. The realized low density used here is consistent with "low" densities used in other work with larval salamanders (Metts et al., 2005), although the high density we used is twice as high as in that study. However, Figiel and

Semlitsch ('90) report natural ranges for *A. maculatum* to be from 0.0002 to 0.08 larvae/L, and the high density used in this study is consistent with the upper end of this range.

After adding larvae, each mesocosm was checked weekly during the next two months. Every two weeks, sets of larvae were haphazardly dipnetted in each mesocosm to monitor larval development (larvae were visually inspected and immediately released back into mesocosms). When we began to find larvae nearing metamorphosis, we switched from weekly to daily monitoring, where we visually checked each mesocosm for individuals that had *finished* metamorphosis (i.e. their gills had been resorbed). Metamorphosed individuals were dipnetted from the mesocosm, placed in a plastic container with pond water, and transported immediately to the lab. It is important to point out here that the hematological approach to estimating stress is not as time-sensitive as direct corticosterone sampling (Davis et al., 2008), where animals must be sampled within minutes of capture (Romero and Reed, 2005; Romero and Romero, 2002). In fact, in amphibians the time for the hematological effect of corticosterone to occur is on the order of hours to days (Bennett and Newell, '65; Bennett et al., '72), which means that any potential effects of capture, handling, or transport are minimal if blood is obtained the same day of capture.

### *Processing adults*

Later in the day of capture, each individual was photographed from above with a digital camera, weighed, and then euthanized by overdose of MS-222. Immediately after death, salamanders were decapitated and a heparinized microcapillary tube was used to siphon blood from the exposed heart region following previously established procedures (Davis and Maerz, 2008a,b). A drop of blood was placed on a clean microscope slide, and a second slide was used to smear the blood on the first slide. All slides were air-dried, and then stained with giemsa. We were only able to obtain enough blood for a readable smear from 30 individuals, although there were enough individuals sampled in each mesocosm to obtain mesocosm-averages (the unit of replication for analyses, below) for blood parameters. Further, the individuals that were not sampled were evenly divided across treatments. Later, body length measurements of all salamanders were obtained from their digital photos using image analysis software following

Davis and Maerz (2007). From these data and the body mass data, we created a body condition score for each salamander by retaining the residuals of a linear regression of the cubed-root of mass on body length.

### *Leukocyte counting*

Counting procedures generally followed Davis and Maerz (2008a,b). All slides were viewed using a light microscope at  $1,000\times$  magnification and the numbers of all white blood cell types were recorded until at least 100 cells were counted or 150 fields of view were reached. Fields of view with low numbers of red blood cells were not included. White blood cell types that we identified included neutrophils, lymphocytes, eosinophils, basophils, and monocytes, following Thrall (2004), Turner ('88), and Hadji-Azimi et al. ('87), although the focus here was on neutrophils and lymphocytes. To ensure accurate counts, each slide was read twice (by AKD), blindly, and in nonconsecutive order, and the average of each leukocyte number was used thereafter. The proportions of each leukocyte type were calculated based on the numbers of each type counted and the total number of all types. We calculated the neutrophil-lymphocyte ratio based on their proportions and used the  $\log(+1)$  of this variable in the analyses.

### *Data analysis*

The effect of larval density on all parameters was examined using multiple regression where the mesocosms were the replicates (experimental units), larval density was the single explanatory variable (treated as a continuous variable), and the mean proportion surviving (arcsine square-root transformed), mean development time, mean mass, mean body condition score, and mean N/L ratio ( $\log+1$ ) of surviving salamanders from each mesocosm were the multiple dependent variables. Levene's tests showed all variables were homogeneous across treatments ( $P < 0.05$  for all). This

statistical approach, where the mesocosm mean is the unit of replication, is highly appropriate to address our experimental objectives, since variations in number of salamanders that survive in each mesocosm (which we expected) would not affect the power of the analysis (since the number of mesocosms remained the same). Indeed, only the sensitivity of the mesocosm mean would be affected by variation in the numbers of salamanders sampled per mesocosm. Analyses were conducted using the Statistica 6.1 software package (Statistica, 2003).

## RESULTS

A total of 52 salamanders survived the larval treatments and were measured in this experiment, with 18, 21 and 13 individuals from low medium and high densities, respectively. Counts of leukocytes from all salamanders for which blood samples were obtained ( $N = 30$ ) are presented in Table 1, grouped according to initial larval density. There were 11 individuals sampled in the low-density treatment, 11 in the medium, and 8 in the high-density treatment. While these parameters were not statistically examined (the experimental unit for analysis was the mesocosm mean, below), they do show how the distributions of cell types within individuals changed with larval density. In general, and considering all individuals, lymphocytes, neutrophils, and eosinophils were counted most often, and very few monocytes were seen, which is consistent with prior research on related *Ambystoma* species (Davis and Maerz, 2008a,b; Davis and Durso, 2009). With regard to density, the average percentage of lymphocytes declined with increasing density, from 32 to 24% and finally 18%, in the low, medium, and high densities, respectively, while the percentage of neutrophils increased from 18% in low densities to 23% in the medium density and 50% of all leukocytes in high densities.

Regression analysis revealed that the overall effect of larval density created significant

TABLE 1. Summary of leukocyte profiles of postmetamorphic spotted salamanders from all three densities

Density	<i>N</i>	Lymphocytes	Neutrophils	Eosinophils	Basophils	Monocytes
Low	11	31.7 (4.3)	18.1 (5.4)	25.5 (4.4)	24.2 (5.1)	0.6 (0.2)
Medium	11	23.9 (2.7)	22.7 (4.3)	33.0 (6.0)	19.6 (3.2)	0.9 (0.4)
High	8	17.8 (2.5)	49.9 (4.1)	22.8 (5.5)	9.3 (2.4)	0.2 (0.2)
All	30	25.1 (2.2)	28.2 (3.6)	27.5 (3.1)	18.5 (2.5)	0.6 (0.2)

Shown are the average percentage (SE in parentheses) of each leukocyte type for all salamanders for which blood samples were obtained ( $N = 30$ ) in each density.

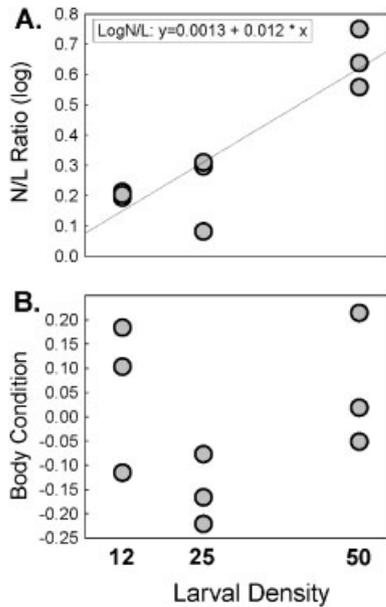


Fig. 1. Relationship between initial larval density and (A) mean neutrophil–lymphocyte ratios (log-transformed) and (B) mean body condition scores (residuals from regression of cubed-root of mass on body length) of surviving salamanders, using the mesocosm mean as the unit of replication.

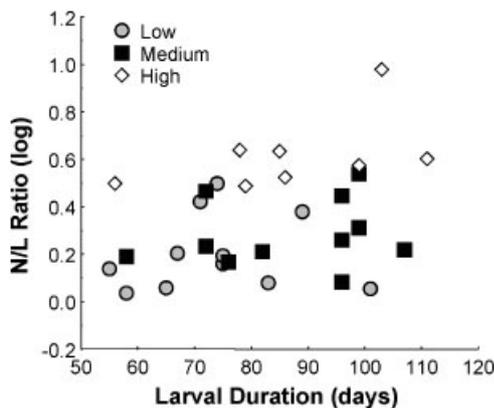


Fig. 2. Neutrophil–lymphocyte ratios (log-transformed) of all salamanders in relation to their larval development time. There was no overall relationship between larval duration and N/L ratios (Pearson correlation,  $r = 0.22$ ,  $P = 0.141$ ), nor was there a relationship when each density was considered separately ( $P > 0.2$  for all).

variation in the parameters evaluated ( $F_{5,3} = 15.80$ ,  $P = 0.023$ ). Consistent with the percentages reported above, there was a significant positive relationship between larval density and mesocosm-mean neutrophil–lymphocyte ratios ( $F_{1,7} = 29.21$ ,  $P = 0.001$ ; Fig. 1A). This relationship was not driven by differences in body condition across densities, as this variable was not significant ( $F_{1,7} = 0.13$ ,  $P = 0.730$ ; Fig. 1B). Nor was the effect of larval density on N/L ratios was not influenced by development time; using data from all individual salamanders, there was no overall relationship between larval duration and N/L ratios (Pearson correlation,  $r = 0.22$ ,  $P = 0.141$ ; Fig. 2), nor was there a relationship when each density was considered separately ( $P > 0.2$  for all). Thus, salamanders in the low-density mesocosms emerged with low N/L ratios regardless of whether they metamorphosed early or late in the experiment, and vice versa for the high density; salamanders in high larval densities emerged with high ratios regardless of their larval duration.

There were effects of rearing density on other, conventional, parameters as well, which support the idea that increasing density is stressful. There was a significant negative relationship between larval density and survival ( $F_{1,7} = 37.40$ ,  $P < 0.001$ ; Table 2), with the highest survival in the low-density treatments (averaging 50% across all three replicate mesocosms), 28% in the medium density, and 8.7% in the high-density treatments. In addition, weights of surviving salamanders decreased with increasing larval densities ( $F_{1,7} = 43.05$ ,  $P < 0.001$ ); salamanders from the low-density treatments weighed an average of 701.9 mg, those from the medium density treatments weighed 513.0 mg, and high-density individuals weighed 324.5 mg on average (Table 2). There was no significant relationship between larval density and development time ( $F_{1,7} = 2.46$ ,  $P = 0.161$ ).

## DISCUSSION

In this experiment, our three larval densities effectively created rearing environments that

TABLE 2. Summary of larval survival, duration, and weights of surviving salamanders from all densities in this experiment

Initial density	N (mesocosms)	Survival	Larval duration (days)	Salamander mass (mg)
12	3	50.0% (4.8%)	71.3 (4.1)	701.9 (63.7)
25	3	28.0% (6.9%)	89.1 (4.4)	513.0 (17.9)
50	3	8.7% (1.8%)	86.2 (4.8)	324.5 (8.7)

Values shown are the grand means of each mesocosm mean, with standard errors in parentheses. Each mesocosm contained 650 L of water.

varied in their quality as shown by two of the traditional measures of larval performance: salamanders reared in the highest larval densities had the poorest survival and lowest masses at metamorphosis compared with the lowest densities, which is consistent with many prior studies (e.g. Wilbur, '76; Collins, '79; Semlitsch and Caldwell, '82; Werner, '86; Petranka, '89; Altwegg and Reyer, 2003; Loman, 2004). Importantly, we show here for the first time that this variation in environmental quality also affects the stress levels of recently metamorphosed salamanders that survive these "stressful" conditions, as determined from neutrophil-lymphocyte ratios, which are known to reflect stress hormone levels in amphibians (Bennett and Harbottle, '68; Bennett et al., '72; Bennett, '86; Davis and Maerz, 2008a,b), birds (Gross and Siegel, '83), and other animals (reviewed in Davis et al., 2008). In the highest larval density, those individuals that successfully survived had the highest proportion of neutrophils, the lowest proportion of lymphocytes, and the highest N/L ratios (i.e. highest stress levels). Further, it did not matter whether they metamorphosed early or late in the experiment, the degree of stress was the same after they emerged (Fig. 2).

The lack of an effect of larval duration on stress levels of postmetamorphic salamanders deserves further comment, since it is somewhat counter-intuitive to conventional ideas of density effects. Specifically, the effects of high densities on individual fitness are thought to be more pronounced early in development when many larvae are present, since as time goes on and larvae metamorphose (or die from other factors), fewer individuals remain and competition for resources is lessened, leading to "density-mediated compensation" in growth or survival (Rohr et al., 2006). Thus, one might initially expect later-emerging salamanders to have lower stress than early emerging individuals. Since our results do not support this idea, we interpret this to mean that either some other factor besides food resources or competition influences stress levels in amphibians, or that the effect of the *initial* density on amphibian stress levels is long-lasting among individuals.

The variation in stress indices we observed among densities did not appear to be caused by variation in nutritional condition of salamanders, since surviving individuals did not vary significantly in body condition across larval densities (Fig. 1B). In high density conditions (especially

early in development), there is considerable competition for food resources, which usually leads to reductions in body size (e.g. Wilbur, '76; Steinwascher, '79; Travis, '84; Petranka, '89), which we did see, but it is also possible that this increased competition leads to increased aggression among larvae (Semlitsch and Reichling, '89; Reques and Tejado, '96; Faragher and Jaeger, '98) and therefore higher stress levels. This idea has certainly been shown before in leopard frog larvae, with higher densities and/or reduced food availability, leading to higher levels of corticosterone among individuals (Glennemeier and Denver, 2002b; Crespi and Denver, 2005). Interestingly though, Rot-Nikcevic et al. (2006) also demonstrated that amphibian larvae can become stressed even if they simply perceive high densities (i.e. in that case by the sight of larvae reflected in mirrors), when in fact the real density is not high. It may well be then that it is not physical competition per se that leads to stress in individuals, but merely the perception of it.

While we do not know how long the elevated stress in salamanders lasts after they leave the aquatic environment, we can infer from studies of other taxa what the consequences would be if it does persist. High ratios of neutrophils (or heterophils in birds) to lymphocytes is often correlated with reduced growth (Moreno et al., 2002), survival (Kilgas et al., 2006), reproductive success (Al-Murrani et al., 2006), and increased susceptibility to infections (Al-Murrani et al., 2002). In amphibians, high stress hormone levels in adults lead to reductions in foraging success (Watson et al., 2004) and increased susceptibility to trematode infections in larvae (Belden and Kiesecker, 2005). Thus, if the high hematological stress parameters we observed in recently metamorphosed salamanders persist, some of these consequences could be realized in the long-term. Interestingly, evidence that low-quality larval environments leads to reduced survival and reproductive success in adult salamanders (Scott, '94) is consistent with effects of chronic stress. Moreover, recent work with anurans demonstrated that experimental exposure of tadpoles to corticosterone lead to juvenile frogs with elevated corticosterone 2 months later (Hu et al., 2008). While these studies all point to the potential for stress to be long-lasting in amphibians, longer-term studies of stress levels of marked individuals would be needed to more clearly elucidate the temporal persistence of stress induced from larval environments.

Finally, besides the question of temporal persistence, our results engender further questions regarding effects of other low-quality larval environments such as wetlands contaminated with agricultural or industrial chemicals. Indeed, while the effects of such chemicals on amphibians are currently being considered (e.g. Rohr and Crumrine, 2005; Forson and Storfer, 2006), the possibility that such larval conditions could lead to stressed metamorphic individuals has not yet been addressed. Given the results from this study, we might expect this to be true. In any case, our results at the very least show that physiological stress can be considered one more example of the legacy that postmetamorphic amphibians carry over from their larval environments.

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### LITERATURE CITED

- Al-Murrani WK, Al-Rawi IK, Raof NM. 2002. Genetic resistance to *Salmonella typhimurium* in two lines of chickens selected as resistant and sensitive on the basis of heterophil/lymphocyte ratio. *Br Poult Sci* 43:501–507.
- Al-Murrani WK, Al-Rawi AJ, Al-Hadithi MF, Al-Tikriti B. 2006. Association between heterophil/lymphocyte ratio, a marker of ‘resistance’ to stress, and some production and fitness traits in chickens. *Br Poult Sci* 47:443–448.
- Altwegg R, Reyer HU. 2003. Patterns of natural selection on size at metamorphosis in water frogs. *Evolution* 57:872–882.
- Beck CW, Congdon JD. 2000. Effects of age and size at metamorphosis on performance and metabolic rates of Southern Toad, *Bufo terrestris*, metamorphs. *Funct Ecol* 14:32–38.
- Belden LK, Kiesecker JM. 2005. Glucocorticosteroid hormone treatment of larval treefrogs increases infection by *Alaria* sp trematode cercariae. *J Parasitol* 91:686–688.
- Bennett MF. 1986. Stress and changes in the blood of newts, *Notophthalmus viridescens*, during early regeneration. *J Comp Physiol A* 159:823–826.
- Bennett MF, Harbottle JA. 1968. The effects of hydrocortisone on the blood of tadpoles and frogs, *Rana catesbeiana*. *Biol Bull* 135:92–95.
- Bennett MF, Newell NC. 1965. A further study of the effects of hydrocortisone on the blood of frogs. *Va J Sci* 16:128–130.
- Bennett MF, Gaudio CA, Johnson AO, Spisso JH. 1972. Changes in the blood of newts, *Notophthalmus viridescens*, following administration of hydrocortisone. *J Comp Physiol A* 80:233–237.
- Collins JP. 1979. Intra-population variation in the body size at metamorphosis and timing of metamorphosis in the bullfrog, *Rana catesbeiana*. *Ecology* 60:738–749.
- Crespi EJ, Denver RJ. 2005. Roles of stress hormones in food intake regulation in anuran amphibians throughout the life cycle. *Comp Biochem Physiol A* 141:381–390.
- Davis AK. 2009. Metamorphosis-related changes in leukocyte profiles of larval bullfrogs (*Rana catesbeiana*). *Comp Clin Pathol* 18:181–186.
- Davis AK, Durso AM. 2009. Leukocyte differentials of northern cricket frogs (*Acris c. crepitans*) with a compilation of published values from other amphibians. *Herpetologica*.
- Davis AK, Maerz JC. 2007. Spot symmetry predicts body condition in spotted salamanders, *Ambystoma maculatum*. *Appl Herpetol* 4:195–205.
- Davis AK, Maerz JC. 2008a. Comparison of hematological stress indicators in recently captured and captive paedomorphic mole salamanders, *Ambystoma talpoideum*. *Copeia* 2008:613–617.
- Davis AK, Maerz JC. 2008b. Sex-related differences in hematological stress indices of breeding, paedomorphic mole salamanders. *J Herpetol* 42:197–201.
- Davis AK, Maney DL, Maerz JC. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Funct Ecol* 22:760–772.
- Dhabhar FS, Miller AH, McEwen BS, Spencer RL. 1995. Effects of stress on immune cell distribution—dynamics and hormonal mechanisms. *J Immunol* 154:5511–5527.
- Dhabhar FS, Miller AH, McEwen BS, Spencer RL. 1996. Stress-induced changes in blood leukocyte distribution—role of adrenal steroid hormones. *J Immunol* 157:1638–1644.
- Faragher SG, Jaeger RG. 1998. Tadpole bullies: examining mechanisms of competition in a community of larval anurans. *Can J Zool* 76:144–153.
- Figiel CR, Semlitsch RD. 1990. Population variation in survival and metamorphosis of larval salamanders (*Ambystoma maculatum*) in the presence and absence of fish predation. *Copeia* 818–826.
- Forson D, Storfer A. 2006. Atrazine increases ranavirus susceptibility in the tiger salamander, *Ambystoma tigrinum*. *Ecol Appl* 16:2325–2332.
- Gervasi SS, Foufopoulos J. 2008. Costs of plasticity: responses to desiccation decrease post-metamorphic immune function in a pond-breeding amphibian. *Funct Ecol* 22:100–108.
- Glennemeier KA, Denver RJ. 2002a. Developmental changes in interrenal responsiveness in anuran amphibians. *Integr Comp Biol* 42:565–573.
- Glennemeier KA, Denver RJ. 2002b. Role for corticoids in mediating the response of *Rana pipiens* tadpoles to intraspecific competition. *J Exp Zool* 292:32–40.
- Gross WB, Siegel HS. 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. *Avian Dis* 27:972–979.
- Hadji-Azimi I, Coosemans V, Canicatti C. 1987. Atlas of adult *Xenopus laevis laevis* hematology. *Dev Comp Immunol* 11:807–874.
- Hu F, Crespi EJ, Denver RJ. 2008. Programming neuroendocrine stress axis activity by exposure to glucocorticoids during postembryonic development of the frog, *Xenopus laevis*. *Endocrinology* 149:5470–5481.
- Hull KL, Cockrem JF, Bridges JP, Candy EJ, Davidson CM. 2007. Effects of corticosterone treatment on growth,

- development, and the corticosterone response to handling in young Japanese quail. *Comp Biochem Physiol A Mol Integr Physiol* 148:531–543.
- Jain NC. 1986. *Schalm's veterinary hematology*. Philadelphia: Lea & Febiger.
- Kiank C, Holtfreter B, Starke A, Mundt A, Wilke C, Schutt C. 2006. Stress susceptibility predicts the severity of immune depression and the failure to combat bacterial infections in chronically stressed mice. *Brain Behav Immun* 20:359–368.
- Kilgas P, Tilgar V, Mand R. 2006. Hematological health state indices predict local survival in a small passerine bird, the great tit (*Parus major*). *Physiol Biochem Zool* 79:565–572.
- Krain LP, Denver RJ. 2004. Developmental expression and hormonal regulation of glucocorticoid and thyroid hormone receptors during metamorphosis in *Xenopus laevis*. *J Endocrinol* 181:91–104.
- Langkilde T, Shine R. 2006. How much stress do researchers inflict on their study animals? A case study using a scincid lizard, *Eulamprus heatwolei*. *J Exp Biol* 209:1035–1043.
- Loman J. 2004. Density regulation in tadpoles of *Rana temporaria*: a full pond field experiment. *Ecology* 85:1611–1618.
- Martin LB, Gilliam J, Han P, Lee K, Wikelski M. 2005. Corticosterone suppresses cutaneous immune function in temperate but not tropical House Sparrows, *Passer domesticus*. *Gen Comp Endocrinol* 140:126–135.
- Metts BS, Hopkins WA, Nestor JP. 2005. Interaction of an insecticide with larval density in pond-breeding salamanders (*Ambystoma*). *Freshw Biol* 50:685–696.
- Moreno J, Merino S, Martinez J, Sanz JJ, Arriero E. 2002. Heterophil/lymphocyte ratios and heat-shock protein levels are related to growth in nestling birds. *Ecoscience* 9:434–439.
- Petranka JW. 1989. Density-dependent growth and survival of larval *Ambystoma*: evidence from whole-pond manipulations. *Ecology* 70:1752–1767.
- Reques R, Tejedo M. 1996. Intraspecific aggressive behaviour in fire salamander larvae (*Salamandra salamandra*): the effects of density and body size. *Herpetol J* 6:15–19.
- Rohr JR, Crumrine PW. 2005. Effects of an herbicide and an insecticide on pond community structure and processes. *Ecol Appl* 15:1135–1147.
- Rohr JR, Sager T, Sesterhenn TM, Palmer BD. 2006. Exposure, postexposure, and density-mediated effects of atrazine on amphibians: breaking down net effects into their parts. *Environ Health Persp* 114:46–50.
- Rollins-Smith LA, Barker KS, Davis AT. 1997. Involvement of glucocorticoids in the reorganization of the amphibian immune system at metamorphosis. *Dev Immunol* 5:145–152.
- Romero LM, Reed JM. 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? *Comp Biochem Physiol A Mol Integr Physiol* 140:73–79.
- Romero LM, Romero RC. 2002. Corticosterone responses in wild birds: the importance of rapid initial sampling. *Condor* 104:129–135.
- Rosenkilde P, Sorensen I, Ussing AP. 1995. Amphibian hematology: metamorphosis-related changes in blood cells. *Netherlands J Zool* 45:213–215.
- Rot-Nikcevic I, Taylor CN, Wassersug RJ. 2006. The role of images of conspecifics as visual cues in the development and behavior of larval anurans. *Behav Ecol Sociobiol* 60:19–25.
- Scott DE. 1994. The effect of larval density on adult demographic traits in *Ambystoma opacum*. *Ecology* 75:1383–1396.
- Scott DE, Casey ED, Donovan MF, Lynch TK. 2007. Amphibian lipid levels at metamorphosis correlate to post-metamorphic terrestrial survival. *Oecologia* 153:521–532.
- Semlitsch RD, Caldwell JP. 1982. Effects of density on growth, metamorphosis, and survivorship in tadpoles of *Scaphiopus holbrooki*. *Ecology* 63:905–911.
- Semlitsch RD, Reichling SB. 1989. Density-dependent injury in larval salamanders. *Oecologia* 81:100–103.
- Semlitsch RD, Scott DE, Pechmann JHK. 1988. Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. *Ecology* 69:184–192.
- Statistica. 2003. *Statistica version 6.1*, Statsoft Inc.
- Steinwascher K. 1979. Competitive interactions among tadpoles: responses to resource level. *Ecology* 60:1172–1183.
- Thrall MA. 2004. Hematology of amphibians. In: Thrall MA, Baker DC, Lassen ED, editors. *Veterinary hematology and clinical chemistry: text and clinical case presentations*. Philadelphia, PA: Lippincott Williams & Wilkins.
- Travis J. 1984. Anuran size at metamorphosis—experimental test of a model based on intraspecific competition. *Ecology* 65:1155–1160.
- Turner RJ. 1988. Amphibians. In: Rawley AF, Ratcliffe NA, editors. *Vertebrate blood cells*. Cambridge: Cambridge University Press. p 129–209.
- Watson RT, Mathis A, Thompson R. 2004. Influence of physical stress, distress cues, and predator kairomones on the foraging behavior of Ozark zigzag salamanders, *Plethodon angusiclavius*. *Behav Process* 65:201–209.
- Werner EE. 1986. Amphibian metamorphosis: growth rate, predation risk, and the optimal size at transformation. *Am Nat* 128:319–341.
- Wilbur HM. 1976. Density-dependent aspects of metamorphosis in *Ambystoma* and *Rana sylvatica*. *Ecology* 57:1289–1296.
- Wingfield JC, Romero LM. 2001. Adrenocortical responses to stress and their modulation in free-living vertebrates. In: McEwen BS, Goodman HM, editors. *Handbook of physiology, section 7: the endocrine system, vol. IV: coping with the environment: neural and endocrine mechanisms*. New York: Oxford University Press. p 211–234.