Journal of Herpetology, Vol. 45, No. 1, pp. 40–44, 2011

Assessing Stress Levels of Captive-Reared Amphibians with Hematological Data: Implications for Conservation Initiatives

ANDREW K. DAVIS[1] AND JOHN C. MAERZ

Warnell School of Forestry and Natural Resources, The University of Georgia, Athens, Georgia 30602 USA

ABSTRACT.—**Larval amphibians are increasingly being reared for conservation initiatives to bolster declining populations. Few researchers, however, have asked whether reared individuals are functionally equivalent to their wild counterparts. Compared with those in the wild, amphibians reared in captivity may develop in relatively stress-free environments, because they are usually fed ad libitum, raised in the absence of predators and pathogens and in controlled environments. Thus, with few challenges throughout development, would their resting stress levels or reactions to acute stressors be normal? We addressed this question by rearing *Litobates sphenocephalus* and *Ambystoma opacum* from eggs and 10-day-old larvae through to late larval stages in artificial pond environments and by determining their ratios of neutrophils (N) to lymphocytes (L) (two leukocytes that covary with stress hormones) before and after a standardized stressor. We obtained similar samples from wild-caught larvae of equivalent developmental stages and from the same source pond. Baseline and stress-induced N:L ratios of reared *L. sphenocephalus* were statistically similar to those of wild individuals. In contrast, baseline N:L ratios of reared *A. opacum* were slightly higher than those of wild individuals. In general, the magnitude of the leukocyte response to stress for both species (a 3-fold increase in N:L over baseline), was similar to that of wild individuals, suggesting that captive-reared amphibians are capable of mounting a normal physiological stress response. Although this last point provides support for the use of captive-rearing for conservation and research purposes, the unusually high baseline N:L ratios of reared salamanders will require additional research to determine the functional meaning.**

One of the most important conservation issues today is the global decline in amphibian populations (e.g., Daszak et al., 1999; Lips et al., 2005; Kriger and Hero, 2009). Although the causes of these declines may be varied and complex (e.g., Rohr et al., 2008; D'Amen and Bombi, 2009; Kerby et al., 2009), a common management response to these declines is to establish captive colonies or captive-rearing and reintroduction initiatives to save or help bolster declining populations (Bloxam and Tonge, 1995; Griffiths and Pavajeau, 2008; Gagliardo et al., 2010). In such programs, eggs from captive or wild amphibians are typically hatched in captivity, and aquatic larvae are reared in captivity for release into the wild or to maintain the captive colony. Although these actions are necessary in the face of imminent extinctions, one must ask whether individuals reared in captivity are functionally similar to their wild counterparts (Calisi and Bentley, 2009; Fariaa et al., 2010). Consider that when larvae are reared from an early age in captivity, they are usually raised in artificial ponds, mesocosms, or ''cattle tanks'' (Barnett et al., 2001). Generally, these environments are simple, with no other species competitors, predators, or pathogens present, and high-quality food is readily available (usually ad libitum), although some managers do aim to mimic natural abiotic conditions (Essner and Suffian, 2010). Importantly, although most rearing environments are primarily designed to maximize survival and to provide the greatest number of metamorphosed animals, animals that are not ''naturally challenged'' during their development may not function appropriately when eventually released into the wild. This leads to an important question for amphibian-rearing projects: Would captive-raised larvae reared in relatively stress-free environments respond normally to a stressor when released into the wild?

Like all vertebrates, amphibians should have a physiological reaction to stressful events that develops at an early stage of life and that is ultimately intended to promote survival during stress events. It begins with an increased production of glucocorticoid hormones (corticosterone in amphibians) in the bloodstream (Moore and Jessop, 2003). This hormone then orchestrates a cascade of events in the body that prepares the animal for coping with the stressor (reviewed in Wingfield and Romero, 2001). One of these events is a temporary redistribution of circulating leukocytes (white blood cells), which is thought to shunt certain cell types to where they would most be needed during the stress period, such as to the epidermis to fight infections and help with wound closing (Dhabhar et al., 1994, 1996). In this case, high levels of glucocorticoids cause an increase in the proportion of neutrophils and a decrease in the proportion of lymphocytes in circulation, such that the ratio of the two cells (neutrophil:lymphocyte [N:L] ratio) is positively associated with the magnitude of the hormonal increase (reviewed in Davis et al., 2008b). This ratio therefore is useful for assessing the degree of stress that amphibians incur under a variety of circumstances (Davis and Maerz, 2008a,b, 2009; Davis and Maerz, 2010).

Here, we report the results of an experiment that was designed to ask whether captive-reared amphibians have different baseline, or stress-induced, N:L ratios than their wild-reared counterparts. Using two nonthreatened amphibian species from the southeastern United States, Southern Leopard Frogs (*Lithobates sphenocephalus*) and Marbled Salamanders (*Ambystoma opacum*), we reared sets of larvae through to a late developmental stage (in a manner typical of conservation initiatives) and compared the magnitude of their baseline and stress-induced (i.e., after submitting the animals to a standardized stressor) N:L ratios with those of wild individuals. The results of this experiment should provide amphibian conservation practitioners with important information regarding the ability of captive-reared amphibians to cope with real-world stressors.

MATERIALS AND METHODS

Rearing Amphibians.—On 16 January 2009, we collected (via dipnetting) ≥150 *A. opacum* larvae from an ephemeral pond near the University of Georgia campus in Athens, Georgia. This pond had been dry for most of the prior year and had filled after heavy rains just 2 weeks before collecting these larvae (Davis, pers. obs.). Because females of this species lay eggs on the bottom of dry ponds and eggs hatch when the pond fills (Noble and Brady, 1933; Conant and Collins, 1998), we are confident that all collected larvae were approximately 10–14 days old. Collected larvae were returned to the lab and placed in two 40-L aquaria for temporary storage. On 24 January, the larvae were transferred to four 1,000-L UV-

[1] Corresponding Author. Present address: Odum School of Ecology, The University of Georgia, Athens, Georgia 30602 USA; E-mail: akdavis@uga.edu

resistant polyethylene aquaculture tanks that had each been filled with 900 L of dechlorinated tap water and enough leaf litter to cover the bottom. Each bin received 25 larvae total; based on prior work with *Ambystoma* salamanders, this amount represents a moderately low stocking density and should result in minimal crowding stress (Davis and Maerz, 2009). Moreover, prior study showed that higher densities than this lead to low survival in *Ambystoma* larvae (Davis and Maerz, 2009). Bins were situated outdoors near our field lab, and they were left uncovered.

On 15 February, we collected ≥10 clutches of *L. sphenocephalus* eggs from the same pond from which the salamander larvae were collected. Collected eggs were brought to the lab, where they were placed into aquaria filled with dechlorinated tap water (one clutch per aquarium) until hatching. Hatching of all eggs was completed by 22 February. On 2 March, we selected five of the most advanced clutches and haphazardly selected larvae that were transferred to four additional 1,000-L bins. As described above, bins were filled to 900 L with dechlorinated tap water, and a layer of leaf litter covered their bottoms. Each bin received 24 larvae from each of the five clutches (120 total larvae per bin). This higher density of Leopard Frog larvae was based on prior studies in our lab that showed very high survival of this species (>80%) at even greater densities than the density used here (Maerz, unpubl. data). Excess larvae that were not selected for study were returned to the source pond.

All rearing bins were monitored daily and food was added weekly. Food for larval frogs consisted of Reptomin floating food sticks, in amounts proportional to the size of the larvae, and there was always enough to ensure their supply did not run out over the week (i.e., their food was provided ad libitum). Salamander larvae were fed zooplankton collected from nearby ponds via a plankton net and by the natural colonization of insects such as mosquitoes. As with frog larvae, the amount of zooplankton provided was proportional to the size of the larvae, and we aimed to ensure the larvae were never without food. Larval frogs and salamanders in the original pond were monitored weekly (via random dipnetting) to gauge their developmental progress. Here, we were only interested in determining when larvae were large enough to sample (see below).

Sampling and Stress Procedures.—On 9 April, 50 larval *A. opacum* salamanders were collected from the original pond. We noted that there were no unusual weather events in the vicinity of the pond during the prior 10 days (i.e., weather that might have influenced salamander stress levels). At this time, larvae were approximately 5–7 cm in length and had fully formed gills. Larvae were immediately transported to the lab (10 min away). There, they were divided into two groups of 25. The first group was processed immediately and entailed euthanization via a 2% solution of neutral buffered MS-222, weighing with an electronic balance, and obtaining a blood sample following Davis and Maerz (2008a,b, 2009). Standard blood films were made for each salamander. We considered the data from these initial blood films to represent ''baseline'' leukocyte data (see below), because the effects of capture, handling, and transport are negligible on N:L ratios because the time lag between stress-induced glucocorticoid increase and subsequent leukocyte redistribution in amphibians and other ectotherms is typically 24 h (Bennett and Alspaugh, 1964; Bennett and Newell, 1965; Bennett and Harbottle, 1968; Bennett et al., 1972; Aguirre et al., 1995), which leaves ample time for obtaining initial, baseline blood samples.

Salamander larvae from the second group were placed individually into 1-L plastic containers that had been filled with dechlorinated tap water. Then, each salamander was gently agitated by hand for 1 min, similarly to the 30-sec ''chase'' procedure used in Langkilde and Shine (2006) on captive skinks. Thereafter, the salamanders were left undisturbed for 24 h, a time long enough to allow glucocorticoid-induced changes to leukocytes (reviewed in Davis et al., 2008b). After this time, they were removed and processed following the procedures described above. This group is hereafter referred to as the ''stressed'' treatment. We point out that the salamander larvae in this group no doubt perceived the capture alone as stressful (Davis and Maerz, 2010), and as such it may not have been necessary to even perform the agitation procedure. However, because the object was to elucidate the leukocyte stress response of reared and wild larvae, we felt it necessary to ensure all larvae underwent an additional, standardized stressor besides capture. Finally, the entire process was repeated for a second batch of 45 *A. opacum* larvae later that week, so that in the end, 95 wild salamander larvae were examined (45 larvae in the baseline group and 50 in the stressed group).

At the time the wild salamander larvae were examined, larvae in the rearing bins were not as developed, so we waited an additional 20 days for them to reach a similar size before we sampled the captive-reared larvae. On that day, all surviving salamander larvae from the rearing bins were removed and transported to the lab. There were 11, 20, 14, and 11 larvae from the four bins (56 total), and the larvae from each bin were divided into the two treatment groups, so that each bin was represented in both treatments (27 in the baseline group and 29 in the stressed group). The first group was processed immediately, whereas the second group underwent the stress procedure outlined above and was processed 24 h later.

On 19 April and 7 May, 56 larval Leopard Frogs in total were collected from the same pond and brought to the lab where similar procedures were performed. Again, there were no unusual weather events noted during the prior 10 days of these dates. At this time, most larvae were between Gosner (1960) stages 30–39. No larvae were beyond stage 39, so all larvae were premetamorphosis. Over the two sampling periods, 30 of these larvae were examined immediately after arrival (i.e., for baseline samples), and 17 larvae were subjected to the stress procedure (placement into individual containers, agitation for 1 min, and sampling 24 h later). From 20 to 25 April, frog larvae from the rearing bins were collected for the same procedures. Because survival of Leopard Frog larvae was much higher than *Ambystoma* larvae (survival was between 77 and 83% in our four frog bins), we haphazardly selected 40 frog larvae from each bin for study. These larvae ranged in Gosner stage from 29 to 35. Upon arrival to the lab, each set of 40 was divided equally into the two treatment groups and sampled accordingly. Certain larvae were processed, but we were not able to obtain sufficient blood for a film. In the end, 63 frog larvae in total were processed and sampled in the baseline group, whereas 74 frog larvae were sampled in the stressed group.

Leukocyte Counting.—Blood films from frog and salamander larvae were examined with a standard light microscope under × 1,000 magnification (oil), following procedures used previously (Davis, 2009a; Davis et al., 2004, 2008a). Only fields of view with relatively uniform cell distributions were examined, and all leukocytes within each field were identified as neutrophils, lymphocytes, eosinophils, basophils, and monocytes following Hadji-Azimi et al. (1987) and Thrall (2004). At least 100 leukocytes were counted for each individual, and from these data we calculated the percentage of all cell types, although we focus here on neutrophils and lymphocytes. The N:L ratios for all individuals were calculated based on the percentages of both cell types.

Data Analyses.—Frog and salamander N:L ratios were log-transformed (+1) to approximate normal distributions. In the salamander data set, there were eight data points that were >2 SDs above the mean for the baseline (four points) and stressed (four points) samples, and these were removed before analyses.

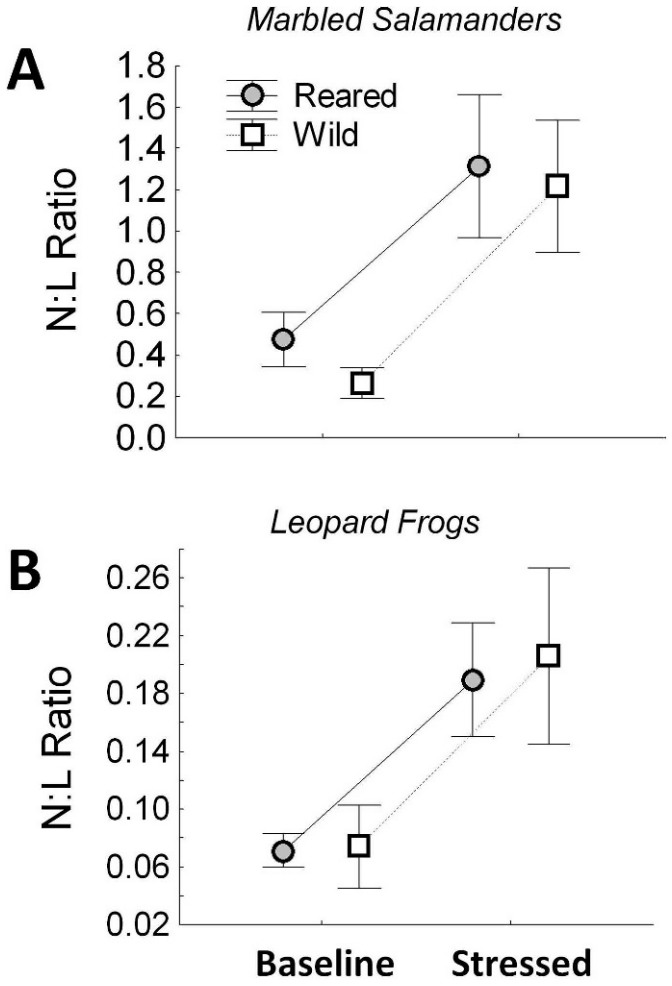

FIG. 1. Average neutrophil:lymphocyte ratios of reared and wild larval Marbled Salamanders (A) and Southern Leopard Frogs (B), examined either immediately after capture (baseline) or 24 h after stress procedure (stressed; see Methods). Error bars represent 95% confidence intervals.

Similarly, there were two outliers in the frog data set that were greater than the mean plus 2 SDs for the baseline (one point) and stressed (one point) samples, and these samples were removed before analyses. There was no significant variation in N:L ratios of frog larvae among bins (one-way analysis of variance: $F_{3,132} = 1.77$, $P = 0.156$), nor was there a bin effect on salamander N:L ratios ($F_{3,48} = 0.93$, $P = 0.434$). We therefore pooled the data from all bins for salamanders and similarly for frogs in further analyses. Then, to address the central question of this study (do captive-reared larvae have different baseline or stressed N:L ratios than wild larvae), we examined transformed N:L ratios of salamanders and frogs by using general linear models, where the larval environment (reared or wild) and sample treatment (baseline or stressed) were categorical explanatory variables, and body mass was a covariate. We also included all two-way interaction terms in the initial models, and we selected the minimum adequate model that contained only significant terms and interactions as outlined by Crawley (2005). All analyses were conducted using the STATISTICA (vers. 6.1, StatSoft, Inc., 2003).

RESULTS

The relative abundance of all five white blood cell types of *A. opacum* and *L. sphenocephalus* differed considerably, especially with respect to the percentage of circulating neutrophils and to a lesser extent, circulating lymphocytes. Nonstressed, wild-caught, larval Marbled Salamanders showed an average neutrophil percentage of 13.6% (±9.6% SD) and an average of 61.0% (±13.8% SD) lymphocytes, which is within the range reported in most other amphibians (Davis and Durso, 2009). In contrast, the average percentage of neutrophils and lymphocytes in wild-caught, nonstressed Leopard Frog larvae was 5.2% (±4.5% SD) and 77.0% (±11.3% SD), respectively. This especially low abundance of neutrophils in Leopard Frog larvae led to much lower overall N:L ratios of larval leopard frogs than marbled salamanders.

There was no support for inclusion of any of the two-way interaction terms in the initial model of N:L ratios of larval marbled salamanders ($P > 0.5$ for all). Of the main effects, body mass was also not significantly related to N:L ratios ($F_{1,137} = 0.11$, $P = 0.739$) and was removed from the model. The final (minimum adequate) model therefore contained the effects of the larval environment (reared or wild: $F_{1,138} = 6.13$, $P = 0.014$) and sample treatment (baseline or stressed: $F_{1,138} = 97.03$, $P < 0.0001$). Thus, regardless of rearing environment, salamanders that underwent the stress treatment had significantly higher N:L ratios than the baseline group, and, salamanders reared in captivity had slightly, but significantly, higher N:L ratios than those from the wild (Fig. 1A).

In the analysis of frog N:L ratios, none of the two-way interaction effects were significant in the initial model ($P > 0.2$ for all), and they were removed. In a model with main effects, the effect of rearing environment (reared vs. wild) was not significant ($F_{1,178} = 0.72$, $P = 0.398$). The minimum adequate model therefore contained weight ($F_{1,179} = 3.99$, $P = 0.047$) and sample treatment (baseline versus stressed: $F_{1,179} = 38.15$, $P < 0.0001$) as explanatory variables. Overall, although the frog larvae subjected to the stress procedure had significantly higher N:L ratios than did those in the baseline group, there was no difference in N:L ratios of reared versus wild frog larvae in either sample treatment (Fig. 1B). In addition, the effect of body mass was negative, such that larger frog larvae tended to have lower N:L ratios overall.

DISCUSSION

The answer to the original question in this experiment, do captive-reared amphibians have similar baseline or stress-induced N:L ratios as wild amphibians, depended on the species. First, the captive-reared Leopard Frog larvae did indeed have statistically similar N:L ratios (both baseline and stress-induced) as their wild counterparts. We are confident in this result because both the wild-caught and captive-reared larvae were of similar developmental stages, and both groups ultimately originated from the same pond. We only examined one species of frog, so generalizing to all species is premature; however, if the results can be generalized, this would speak to the suitability of conservation-related captive-rearing initiatives for producing frogs that have normal resting stress levels and normal reactions to stressful events.

Although tangential to the goals of this study, we also discovered a slight but significant negative relationship with body size and N:L ratio in Leopard Frog larvae. Because we had only intended to statistically control for possible effects of variation in body size, we did not have any a priori predictions regarding the direction of the effect, if any. There is in fact, little research into the relationship between body size and stress physiology in any animal, let alone amphibians. Nevertheless, it seems that larger larvae had lower N:L ratios. Given that all larvae hatched at roughly the same time (within a few days), this pattern may indicate a correlation between rapid growth and lower circulating stress hormones as indicated by N:L ratios. Similar relationships have been reported for nestling

birds, where low ratios were associated with fast growth (Moreno et al., 2002).

Results concerning N:L ratios of larval marbled salamanders differed somewhat from those of frog larvae, and in an unexpected direction. Our analysis of these data suggest that although the captive-reared larvae seemed capable of mounting a stress response of a similar magnitude as their wild-reared counterparts (i.e., their average N:L ratios increased 3-fold from the baseline to the stress group, as did the wild group), their overall N:L ratios were slightly, but significantly higher, not lower, than those from the wild. This was especially pronounced in the baseline group (Fig. 1A). In fact, comparison of only baseline N:L ratios of captive-reared versus wild salamander larvae showed a significant difference (two-sample t-test: $t = 2.64$, df = 66, $P = 0.010$), but a similar comparison of stress-induced N:L ratios showed no significant difference ($t = -1.34$, df = 73, $P = 0.184$). Thus, rather than being ''less stressed'' in the presumably benign rearing environment, captive-reared salamander larvae had higher resting, or baseline N:L ratios than normal, indicating that they had higher baseline levels of corticosterone.

Interestingly, this is not the first case of higher-than-normal resting stress levels found in mesocosm-reared *Ambystoma* salamanders. In a recent study examining the effects of larval density on *A. maculatum*, we found that even in the lowest density used in the experiment (12 larvae initially in 1,000-L bins), salamanders emerged with an average resting N:L ratio of 0.57, nearly twice as high as typical ratios of wild-caught *Ambystoma* salamanders [close to 0.30, Davis, 2009b]). Furthermore, although there are no published comparisons of actual corticosterone level differences between reared and wild salamanders, a recent doctoral project did address this by using *A. jeffersonianum* (Chambers, 2009). In this case, late-stage, mesocosm-reared larvae were found to have moderately elevated resting corticosterone levels compared with wild larvae. In contrast, stress-induced levels in both groups were equivalent (and much higher), consistent with the present study.

Apparently, there is something about the captive-rearing environment that larval *Ambystoma* salamanders perceive as ''mildly stressful.'' One possibility is that because of their carnivorous nature, they may be aggressive toward each other in captivity. Support for this idea comes from an earlier study examining effects of rearing density on rates of tail and appendage injuries of *A. talpoideum* (Semlitsch and Reichling, 1989). Even in the lowest density used in that study, at least 20% of larvae had injuries caused by other larvae, and considerable rates of intraspecific predation and cannibalism were noted. Indeed, even in our experiment, we initially placed 25 larvae into each bin (100 total), but after nearly 4 months, only 56 larvae were left. It is possible that some of these larvae were cannibalized by other larvae. It may be that when in captivity and when there are few other stimuli present, these salamanders act aggressively toward each other; but in the wild, where there is a greater variety of stimuli and prey items, they do not. Regardless, this is an issue that must be considered in conservation-related (captive-rearing) initiatives or research projects involving rearing of similar species, especially those that are carnivorous and might display some degree of intraspecific aggression.

Despite the mildly increased basal N:L ratio of the captive-reared salamanders, it is important to remember that their leukocyte response to stress (i.e., the difference between the basal and stress-induced N:L ratios) was of equal magnitude as their wild counterparts. Thus, both species examined here seemed to show ''normal'' physiological reactions to stress when reared in captivity, which supports the use of captive-rearing for conservation initiatives. However, we do suggest that future research efforts should be aimed at identifying factors that help to minimize stress levels during larval rearing, especially for species that may naturally be aggressive. Given the increasing reliance on captive-rearing and captive-breeding programs to protect declining or endangered amphibian species, this issue should be given a high priority.

Acknowledgments.—We thank the many graduate students and field assistants in the Maerz lab for assistance in the field. We also thank Joe Milanovich for help with agitating the salamanders. AKD was supported by a grant from the Morris Animal Foundation (D08ZO-409). All procedures in this study were approved by the University of Georgia Animal Care and Use Committee (AUC A2010 2-015).

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Accepted: 8 November 2010.