Copeia 2008, No. 3, 613–617

Comparison of Hematological Stress Indicators in Recently Captured and Captive Paedomorphic Mole Salamanders, *Ambystoma talpoideum*

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Measuring stress in animals is an important component of many research studies, and it has traditionally been performed via sampling levels of corticosterone in plasma. A secondary, ''hematological'' approach used most commonly by researchers of birds, mammals, and other taxa involves evaluating leukocyte profiles from blood smears. Such research has shown that leukocytes have a characteristic response to stress, although in amphibians this phenomenon is not as well studied. In general, stress can induce a rise in the ratio of neutrophils to lymphocytes. We evaluated the hematological response of paedomorphic Mole Salamanders (*Ambystoma talpoideum*) to captivity stress, specifically focusing on this parameter, but also examining other white blood cell types. Individuals captured in the wild and held in captivity for ten days before sampling had significantly more neutrophils, fewer lymphocytes, and higher ratios of neutrophils to lymphocytes than those captured from the same locations and sampled within one hour. Captive individuals also had significantly higher numbers of eosinophils. These results are consistent with hematological research in birds and other taxa and highlight the utility of this approach for measuring stress in amphibians.

THE ability to identify physiological stress in animals is important in wildlife research, both in field and laboratory settings. One of the main physiological responses to stressful stimuli in vertebrates is an increase in the plasma levels of glucocorticoid hormones such as corticosterone (Moore and Jessop, 2003; Romero, 2004). This response is characteristic of most taxa including birds (Cockrem and Silverin, 2002), reptiles (Cash et al., 1997), mammals (Lopez-Olvera et al., 2007), and amphibians (Mosconi et al., 2006), and researchers commonly assess plasma levels of these hormones to evaluate the degree of stress an animal is currently under. One drawback to this technique is that the potential for rapid increases in corticosterone (i.e., within minutes) following capture and handling (Romero and Romero, 2002) means that researchers must obtain blood samples from the specimen immediately after capture to obtain ''baseline'' hormone levels.

A secondary approach for assessing physiological stress is via the leukocyte component of the immune system, specifically the relative numbers of white blood cells in circulation. In general, release of stress hormones in vertebrates causes alterations of numbers of two of the five leukocyte types; that is an increase in neutrophils and a decrease in lymphocytes (Jain, 1986, 1993; Dhabhar et al., 1994). The reason for these alterations is not clear, but since it occurs predictably, detection of these alterations can be used by researchers to indirectly infer increases in stress hormones. Moreover, using this approach to measuring stress offers certain benefits to animal researchers, especially in field situations where it may be difficult to sample animals quickly after capture. First, the proliferation of leukocytes in circulation operates on a slower time scale than hormones such that hormone-induced changes in leukocyte numbers are not observed (in birds) within one hour after capture (Davis, 2005); therefore, any possible stress of capture or handling is not reflected in initial (''baseline'') samples. A secondary advantage is the relatively smaller amount of blood required for making a blood smear than for sampling corticosterone in plasma (AKD, pers. obs.).

There is convincing evidence that stress-induced hematological alterations (i.e., increases in neutrophils and decreases in lymphocytes) can be seen in nearly all vertebrates. The link between stress hormones and leukocytes has been well documented in mammals (Jain, 1986, 1993; Dhabhar et al., 1994). In birds early work with poultry demonstrated how stress causes the number of heterophils (the neutrophil equivalent in birds and reptiles) to increase while the lymphocytes decrease (Gross and Siegel, 1983). The ratio of these cells has been increasingly used among ornithologists to document the effects of various stressful conditions including transport stress (Groombridge et al., 2004) and reproductive output (Moreno et al., 2002a). Research with Eastern Box Turtles (*Terrapene carolina carolina*) also used this approach to document stress (Case et al., 2005). Furthermore, a series of lab experiments beginning in the 1960s showed that amphibians appear to have the same response, in multiple stress-inducing conditions such as limb amputation (Bennett, 1986), temperature extremes (Bennett and Daigle, 1983), osmotic stress (Bennett and Johnson, 1973), photoperiod stress (Bennett and Reap, 1978), and direct administration of hydrocortisone (Bennett and Alspaugh, 1964; Bennett and Newell, 1965; Bennett and Harbottle, 1968; Bennett et al., 1972). In all cases, the stressors caused increases in numbers of neutrophils and decreases in lymphocyte numbers in circulation. Unfortunately, a thorough review of more recent literature revealed that this early research has not been subsequently followed, nor has this ''hematological'' stress assay been used in recent studies of amphibians. The one exception is a recent study conducted

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Submitted: 13 June 2007. Accepted: 4 December 2007. Associate Editor: E. Schultz.
 DOI:

by the authors of the current paper where neutrophil–lymphocyte ratios of breeding male and female Mole Salamanders (*Ambystoma talpoideum*) were used to assess sex-related differences in reproductive stress (Davis and Maerz, 2008).

Short-term captivity is known to induce a hormonal stress response in wild animals (Cash et al., 1997; Mosconi et al., 2006). While this phenomenon is well known, the effects of captivity-induced stress on leukocyte profiles have rarely been examined, and thus far only in birds (Ruiz et al., 2002). Similar research has not been conducted with amphibians, despite the fact that many studies of frogs or salamanders involve captive husbandry. In this study we examined the hematological profile of a common amphibian species (Mole Salamander), captured in the wild and brought into captivity for ten days to test the hypothesis that captivity induces a measurable hematological stress response, reflected specifically by increases in neutrophils, decreases in lymphocytes or increases in neutrophil–lymphocyte ratios. We also examined possible effects of stress on cell counts of the three other leukocyte types (eosinophils, basophils, and monocytes), although since these cells are not known to be affected by stress (Thrall, 2004), we had no *a priori* reason for suspecting an effect of captivity on these cells.

MATERIALS AND METHODS

Capturing salamanders.—We captured paedomorphic Mole Salamanders on two days from three permanent ponds located in the Warnell School of Forestry's Whitehall Experimental Forest in northeast Georgia in March 2007. On the first sampling day (15 March), we dipnetted between five and six salamanders per pond (16 total), then placed them in plastic containers filled with pond water, and transported them to a location 10 km away where they were placed in a 40 L plastic container filled with aged well water. The container was placed in a semi-shaded spot outside, and a small amount of leaf litter was added to provide refugia within the container. These individuals were left undisturbed until 25 March (ten days later), when they were hand-captured and transported to the lab for processing as described below. On the second sampling trip (20 March) we visited the same three ponds, captured 5–6 individuals per pond as before (16 total), but these individuals were immediately transported to the lab for processing. In this case, all individuals were sampled within one hour of capture, including the transport time.

Blood sampling and slide preparation.—In the lab each salamander was weighed then killed via overdose of MS-222. A blood sample was then obtained by siphoning a small sample of blood from the exposed heart region after decapitation with a microhematocrit tube following Davis and Maerz (2008). A drop of the blood was then used to make a standard blood smear. Smears were allowed to air-dry and were then stained with Giemsa.

Leukocyte counting and data handling.—All slides were viewed with a standard light microscope under 1000X (by only one of the authors), and at least 100 leukocytes were counted, while keeping track of the number of fields of view examined following Davis and Maerz (2008) and Davis et al. (2004). During the counting, slides were moved in increments of approximately 1–2 mm (without the observer looking through the lens), in a standard zig-zag pattern across the blood smear, so that all parts of the smear were eventually sampled. While the observer did not view the slide during the incremental moves, if the field of view landed where there was limited coverage of erythrocytes (i.e., less than 15), that field was not used. We identified leukocytes as lymphocytes, neutrophils, eosinophils, basophils, and monocytes following Thrall (2004). Thrombocytes were observed throughout the smears but are not considered leukocytes (Thrall, 2004) and therefore were not counted. When 100 leukocytes were counted, we calculated the number of leukocytes per field of view, based on the number of fields examined.

For each salamander we estimated the total number of leukocytes per 1000 red blood cells by dividing the number of leukocytes per field of view by the average number of erythrocytes per field of view (18 [standard deviation = 5], obtained from a subset of five fields of view from each of ten individuals) and multiplying by 1000. We then calculated the proportion of all five leukocyte types, and estimated the relative numbers of each leukocyte type per 1000 erythrocytes for each salamander by multiplying the cell proportion by the total number of leukocytes per 1000 erythrocytes. Finally, we calculated the ratio of neutrophils to lymphocytes using the proportions of each cell type following Davis and Maerz (2008) and Davis et al. (2004). The ratios were arcsin squareroot transformed and numbers of leukocytes for each cell type were log-transformed prior to analyses to meet assumptions of normality.

Data analysis.—We examined the possible effect of captivity while simultaneously examining possible effects of body size using analysis of covariance with counts of each leukocyte type (i.e., estimates of cell number per 1000 erythrocytes), as well as neutrophil–lymphocyte ratios, included as dependent variables, with treatment (recently captured or captive) and body mass included as independent variables. In all, five analyses were performed (one for each leukocyte type [except monocytes – see results], and one for neutrophil–lymphocyte ratios). We did not include a pond variable since all three ponds were of similar size and located within 50 m of each other and since *a priori* examination of possible differences in all five leukocyte parameters among ponds revealed no significant variation (one-way ANOVA, $df = 2$, $P > 0.05$ for all five parameters). All analyses of covariance were run once with an interaction term between the two independent variables included in the model, but because there was no significant effect of this term found in any analyses, it was removed from the model and the analyses repeated with main effects only. All tests were performed using Statistica 6.0.

RESULTS

Body size.—The size of the salamanders (i.e., body mass) was not significantly related to the neutrophil–lymphocyte ratio, nor to counts of lymphocytes or neutrophils (Table 1). However, body mass significantly affected counts of eosinophils, and evaluation of parameter estimates indicated this effect was positive such that larger salamanders had more eosinophils.

Effect of treatment.—The neutrophil–lymphocyte ratio was significantly higher in the captive-stressed individuals than

Table 1. Summary of Results of ANCOVAs Examining Effects of Body Size (Mass) and Treatment (Recently Captured Versus Captive-Stressed) on Leukocyte Parameters of Paedomorphic Mole Salamanders. Counts of leukocyte types (estimated numbers per 1000 rbcs) were used in analyses of specific cell types. Not enough monocytes were counted to allow for statistical testing. In all tests, an interaction effect was initially included, but was non-significant ($P > 0.05$) in all cases and therefore removed.

Dependent	Independent	df	MS	*F*	*P*
N-L ratio	Wt	1	0.00	0.10	0.749
	Treatment	1	0.52	28.97	0.000
	Error	29	0.02		
Lymphocytes	Wt	1	0.01	0.11	0.739
	Treatment	1	0.24	4.93	0.034
	Error	29	0.05		
Neutrophils	Wt	1	0.02	0.53	0.474
	Treatment	1	0.26	5.67	0.024
	Error	29	0.05		
Eosinophils	Wt	1	0.89	5.33	0.028
	Treatment	1	0.81	4.83	0.036
	Error	29	0.17		
Basophils	Wt	1	0.15	3.40	0.076
	Treatment	1	0.00	0.00	0.955
	Error	29	0.04		

that of the recently captured (Table 1). In terms of captivity effects on individual cell types, captive-stressed salamanders had significantly fewer lymphocytes and significantly more neutrophils and eosinophils (Tables 1, 2) than recently captured salamanders. There was no effect of captivity on numbers of basophils. Not enough monocytes were counted for statistical tests.

DISCUSSION

The hematological approach we used here demonstrated that regardless of body size, captivity induced a general stress response in the paedomorphic Mole Salamander. Specifically, captivity caused the estimated numbers of neutrophils to increase and numbers of lymphocytes to decrease; thus, the ratio of neutrophils to lymphocytes of captive individuals was on average twice as high as that of recently captured individuals. These results are consistent with prior studies of other stress-inducing conditions on amphibian leukocyte parameters (Bennett and Reap, 1978; Bennett and Daigle, 1983; Bennett, 1986), although we point out that the stress-inducing stimulus involved in our study was considerably more benign than those previously studied in amphibians, which included limb amputation and direct injection of stress hormones. Indeed, in our study we did little more than capture wild salamanders and place them in a standard mesocosm-type container filled with water, and they were then left undisturbed for ten days. The hematological parameters we observed after this ten-day period, compared to non-captive individuals, indicated that even this relatively simple procedure induced a physiological stress response in the salamanders.

While it is clear that both sets of salamanders we assessed differed in hematological parameters (and we logically conclude that captivity caused this difference), we cannot completely rule out the possibility that the five-day time difference between sampling the first set (those later held captive for ten days) and the second set was the reason for the observed results, and not necessarily any effect of stress. We consider this possibility unlikely though, since prior collections of ten non-breeding individuals made from one of these same ponds three months earlier revealed similar leukocyte profiles and a statistically similar average neutrophil–lymphocyte ratio to the recently captured individuals of this study (two-sample t-test, $t = 1.69$, $df = 24$, $P = 0.103$). Thus, we argue that if the hematological stress parameters did not change over a three-month period in these ponds, it should not have changed over the five days examined here.

An aspect of this study that evokes further questions is why exactly the stress response was elicited in the captive

Table 2. Summary of Leukocyte Profiles (as % of All White Blood Cells or Estimated Number per 1000 Red Blood Cells) of Recently Captured ($n = 16$) and Captive-Stressed ($n = 16$) Paedomorphic Mole Salamanders. Shown are the means plus standard errors in parentheses. Asterisks indicate significant differences between treatments from ANCOVA results in Table 1. $^{*}P < 0.05$ $^{**}P < 0.001$

	Recently captured		Captive-stressed	
	%	per 1000 rbc	%	per 1000 rbc
Lymphocytes	39.0 (5.3)	23.2 (2.8)	21.5 (3.5)	13.4* (1.1)
Neutrophils	5.7 (0.8)	3.8 (0.8)	8.6 (1.6)	5.2 (0.5)
Eosinophils	51.2 (5.8)	35.6 (6.2)	66.7 (5.4)	68.6* (13.5)
Basophils	3.9 (0.7)	2.4 (0.5)	2.9 (0.5)	2.1 (0.4)
Monocytes	0.1 (0.1)	0.0 (0.0)	0.3 (0.1)	0.2 (0.1)
N-L Ratio	0.17	(0.03)	0.39**	(0.03)

salamanders. We envision two possible explanations that could be examined further in follow-up work. In the first, it may be that the stress of the initial capture and removal from the natural surroundings caused an acute increase in plasma stress hormone (i.e., corticosterone) levels, which then lead to the alterations in leukocyte parameters, and these alterations could have lasted for the duration of the experiment. Indeed, there is evidence that the production of leukocytes in amphibian tissues and migration into circulation requires days (as opposed to hours in mammals) to complete (Hightower, 1978). Alternatively, the stress of captivity (whether from crowding, suboptimal conditions, etc.) may have elicited a more chronic increase in stress hormones which then caused leukocyte alterations. Determining which of these scenarios is of greater importance should help amphibian researchers pinpoint commonly-used procedures that are likely to cause stress.

While we had no *a priori* expectations for how captivity would affect other leukocyte parameters, we found that captivity induced an unexpected increase in numbers of eosinophils. Eosinophil numbers have not previously been reported to change with stress in amphibians, although there is evidence that eosinophils decrease in abundance following stressful stimuli in mammals (Jain, 1986). This cell is thought to be involved in parasitism defense, since general increases occur in response to parasite infections (Thrall, 2004). Interestingly, this cell is also the most abundant leukocyte in paedomorphic ambystomatid salamanders (Ussing and Rosenkilde, 1995; Davis and Maerz, 2008). Following metamorphosis, the proportion of eosinophils declines to less than 10% of leukocytes (Ussing and Rosenkilde, 1995). However, the high proportions of eosinophils are likely not due to paedomorphism itself, since in the obligate paedomorphic Hellbender (*Cryptobranchus alleganiensis*) eosinophils amount to 4.5% of leukocytes in adults (Jerrett and Mays, 1973). Rosenkilde et al. (1995) suggested that the act of metamorphosis partly serves to clear the animal of parasites which explains the dramatic drop in numbers following (induced) metamorphosis, although since a thorough screening of parasites in ambystomatid salamanders has not yet been performed, we can not be sure of this idea.

Our study demonstrates the utility of using hematological techniques to assess stress in amphibians. Our results also have implications for a wide range of herpetological research, but specifically for projects involving captive husbandry of amphibians. In such cases, researchers must be aware that animals brought into captivity from wild sources become stressed, regardless of the experimental conditions being explored. Moreover, stressed animals may not behave or react in a manner typical of normal animals, although this point should be better studied in herpetofauna. We can make some inferences from work with other taxa. In birds, juveniles with high heterophil–lymphocyte ratios grow more slowly to adulthood (Moreno et al., 2002b), and adults with high ratios survive less well from year to year than those with low ratios (Kilgas et al., 2006). Also in birds, individuals with high ratios have been shown to be more susceptible to diseases than those with low ratios (Al-Murrani et al., 2002). Finally, and perhaps more related to captive husbandry, the quality of the housing environment has been shown to be an important predictor of hematological stress parameters in Box Turtles (*Terrapene carolina*) in captivity (Case et al., 2005). These studies, as well as the present one, all highlight the importance of knowing the conditions under which animals become stressed in research projects, and more generally, the influence of stress on immune systems and life history patterns of all vertebrates, including herpetofauna.

ACKNOWLEDGMENTS

AKD was supported by funds from the D. B. Warnell School of Forestry and Natural Resources at the University of Georgia. Procedures in this study were approved by the University of Georgia Institutional Animal Care and Use Committee (AUP A2007-10014-0).

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