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Sex-Related Differences in Hematological Stress Indices of Breeding Paedomorphic Mole Salamanders

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

D. B. Warnell School of Forestry and Natural Resources, University of Georgia, Athens Georgia 30602, USA

ABSTRACT.—Reproduction in amphibians is stressful and perhaps more so in females than males because of the higher energetic costs of producing eggs than sperm. The ratio of two white blood cells, neutrophils and lymphocytes, has been shown in birds, turtles, and amphibians to increase with stress. We captured breeding and nonbreeding, paedomorphic Mole Salamanders (*Ambystoma talpoideum*), to determine whether the stress of reproduction is reflected by neutrophil:lymphocyte ratios in this species. In blood smears of all individuals, we observed approximately 25.5 leukocytes per 1000 erythrocytes, with 13% of the leukocytes being neutrophils, 41% lymphocytes, and 46% eosinophils. Less than 1% of leukocytes were basophils and monocytes. Neutrophil:lymphocyte ratios of gravid females were significantly higher (and also more variable) than those of reproductive males and of nonreproductive individuals, indicating a higher degree of physiological stress in reproductive females. Reproductive males did not have higher ratios than nonreproductive individuals. We found no effect of body size on neutrophil:lymphocyte ratios. Our hematological stress results are consistent with studies of other amphibians where different methods were used and with other taxa.

As in all animals, reproduction in amphibians can be stressful. Many species of amphibians in reproductive states have higher levels of the stress hormone corticosterone than nonreproductive individuals (reviewed in Moore and Jessop, 2003). Further, comparisons of metabolic rates of reproductive and nonreproductive salamanders point to higher energy demands (i.e., ''energetic costs'') in the reproductive state (Finkler and Cullum, 2002). Studies of metabolic rates also indicate sex-related differences in energetic costs of reproduction, which Ryan and Hopkins (2000) suggest is attributable to different energy demands on the sexes during reproduction, because ova require more energy to produce than sperm.

Physiological stress can influence immunnocompetence in animals (e.g., Martin et al., 2005). This influence can be seen by alterations in white blood cell (leukocyte) proportions in response to stress. Specifically, stress changes the relative numbers of two of the five vertebrate leukocyte types, neutrophils (called heterophils in birds and reptiles) and lymphocytes, in the circulating blood. Stress causes neutrophil (or heterophil) proportions to increase and lymphocyte proportions to decrease; thus, the ratio of the two has been used extensively as an index of physiological stress levels in poultry (e.g., Gross and Siegel, 1983) and other bird species (e.g., Davis et al., 2004), as well as in turtles (Case et al., 2005). Further, this ''hematological stress'' assay has distinct advantages over traditional measures of corticosterone levels. Although hormone production can begin within 3 min of capture in birds (Romero and Romero, 2002), leukocyte proliferation operates more slowly, and handling time up to one hour does not affect the heterophil-lymphocyte ratio (Davis, 2005). This could also be true for amphibians, which have neutrophil generation times of between two and five days (Hightower, 1978). In contrast, animals must be sampled nearly immediately after capture to obtain baseline levels of corticosterone (Romero and Romero, 2002). Thus, the hematological stress response reflects more long-term physiological stress than does corticosterone, which can fluctuate considerably. Further, the corticosterone stress response ceases to respond to repeated stimuli over time, whereas the hematological response does not, leading McFarlane et al. (1989) to conclude the latter assay is more reliable.

The same hematological stress response has also been observed in amphibians in laboratory settings, although neutrophil:lymphocyte ratios were not specifically examined. A series of experiments in the 1970s and 1980s showed that the hematological response of captive newts to a variety of stressors (including osmotic stress, temperature stress, injection of hydrocortisone, and limb amputation) was always an increase in the proportion of neutrophils and a decrease in the proportion of lymphocytes in the white blood cell population (Bennett et al., 1972; Bennett and Johnson, 1973; Bennett and Reap,

[1] Corresponding Author. E-mail: akdavis@uga.edu

1978; Bennett and Daigle, 1983). However, this research has not been expanded, despite the potential utility of this hematological stress assay in herpetological research and conservation, especially when anthropogenic stressors to amphibians need to be quantified.

Mole Salamanders (*Ambystoma talpoideum*) are a common amphibian throughout the southeastern United States and are particularly well studied with respect to their tendency toward paedomorphosis in permanent to semipermanent wetlands (e.g., Semlitsch, 1985, 1987). Breeding occurs during the late fall and winter and often while in the paedomorphic state. Further, the amount of parental investment (i.e., size of eggs, testes diameter) during breeding increases with increasing body size (Semlitsch, 1985). Thus, the energetic costs of reproduction appear to increase with body size in this species, which could translate into higher stress for larger breeders.

In this study, we captured male and female paedomorphic *A. talpoideum* that were in reproductive condition and compared their neutrophil:lymphocyte ratios to nonreproductive individuals captured at the same time to determine whether the stress of reproduction is reflected in this hematological parameter. We simultaneously examined potential relationships between neutrophil:lymphocyte ratios and body size. Based on conclusions reached by prior studies of energetic costs of breeding in salamanders, we expected to see higher neutrophil to lymphocyte ratios in reproductive than non-reproductive individuals, reflecting higher physiological stress during breeding, and higher ratios in females than males. We further expected to find positive relationships between body size and neutrophil:lymphocyte ratios.

Materials and Methods

Capturing and Sampling.—Paedomorphic *A. talpoideum* salamanders were dip-netted from a permanent pond near Athens, Georgia, over three days between 30 November and 2 December 2006. A total of 34 individuals were collected, of which 12 were females in reproductive condition (i.e., were gravid), 12 were reproductive males (i.e., had enlarged cloaca), and 10 were nonreproductive individuals of unknown sex. Upon capture, all salamanders were placed individually in 1-liter plastic containers filled with pond water and immediately transported to the lab. Later the same day of capture (i.e., within 3 h), all animals were photographed with a digital camera, killed via immersion in MS-222, and decapitated. A microcapillary tube was used to draw a drop of blood from the exposed heart region, which was spread onto a microscope slide. Although we initially had attempted to obtain blood for this study nondestructively via the caudal vein, we were not successful in obtaining pure blood from this location with a syringe and, therefore, switched to the heart sampling described above. After all animals were sampled, slides were air-dried for 2 h and then stained with a Wright-Giemsa (Quik) stain. From the digital photographs, we measured the snout–vent length (SVL) of each animal using image analysis software following Davis and Maerz (2007).

Leukocyte Counting.—Slides were viewed under 200× with a light microscope. In general, leukocyte counting followed Davis et al. (2004), Davis (2005), and Rutherford et al. (2005) with minor variations. Briefly, at least 100 white blood cells were counted, and the number of fields of view was recorded until 100 white blood cells were reached. Although random fields of view were selected for viewing, only fields of view with even distributions of erythrocytes were used (i.e., where the field was completely filled with cells). We identified cell types as neutrophils, lymphocytes, eosinophils, basophils, and monocytes according to Hawkey and Dennett (1989). The proportion of each leukocyte type was calculated based on the numbers observed, and we calculated the neutrophil:lymphocyte ratio for each individual based on the proportions of each type observed. These values were log-transformed to approximate a normal distribution.

Data Analysis.—Size differences (snout–vent length) among reproductive females, reproductive males, and nonreproductive individuals were analyzed using one-way ANOVA. We used analysis of covariance to examine variation in transformed neutrophil:lymphocyte ratios among reproductive females, reproductive males, and nonreproductive individuals (included as a categorical variable) and to simultaneously examine how this parameter varies with body size (SVL included as a covariate). Analyses were performed using Statistica 6.1 software (2003).

Results

There was an average of 7.23 (± 0.8 SE) white blood cells per field of view (at 200× magnification) in all paedomorphic Mole Salamanders in this study ($N = 34$), which translated to approximately 25.5 leukocytes per 1000 erythrocytes. The average percentage of neutrophils was 13% (± 1.2 SE), of lymphocytes was 41% (± 3.2 SE), and of eosinophils was 46% (± 3.5 SE). We found that less than 1% of all leukocytes were basophils and monocytes.

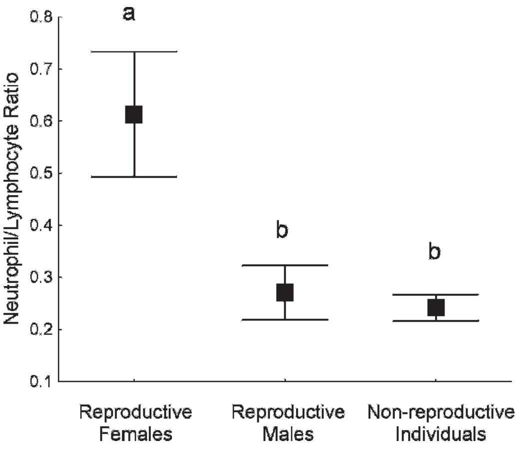

FIG. 1. Neutrophil:lymphocyte ratios of reproductive female, male, and nonreproductive paedomorphic *Ambystoma talpoideum*. Errors bars denote ±1 SE. Letters indicate statistically similar groups, based on Tukey post hoc tests.

Neutrophil:lymphocyte ratios differed significantly between reproductive females, males, and nonreproductive individuals ($F_{2,28} = 3.99$, $P = 0.031$; Fig. 1). Tukey's post hoc comparisons indicated that neutrophil:lymphocyte ratios of reproductive females were significantly greater than that of reproductive males ($P = 0.010$) and nonreproductive individuals ($P = 0.008$). However, neutrophil:lymphocyte ratios of reproductive males did not differ from nonreproductive individuals ($P = 0.974$).

Although there was significant variation in body size among reproductive females, males, and nonreproductive individuals ($F_{2,33} = 14.82$, $P < 0.001$), with average snout–vent lengths of 4.65 (± 0.04 SE) cm, 4.82 (± 0.04 SE) cm, and 4.98 (± 0.04 SE) cm for reproductive females, males, and nonreproductive individuals, respectively, there was no significant effect of body size ($F_{1,33} = 0.19$, $P = 0.666$) on neutrophil:lymphocyte ratios in our ANCOVA. Furthermore, direct comparisons of SVL with neutrophil:lymphocyte ratios within sexes revealed no relationship in females ($r = 0.04$, $P = 0.904$), males ($r = 0.27$, $P = 0.405$), or nonreproductive individuals ($r = -0.26$, $P = 0.462$).

DISCUSSION

Results from this study indicate that female paedomorphic Mole Salamanders experience higher levels of stress during reproduction than do males, based on their higher neutrophil:lymphocyte ratios than reproductive males (Fig. 1). Therefore, this result is consistent with prior studies of sex-differences in amphibian metabolic rates, which indicated a higher energy demand by females than males during reproduction (Ryan and Hopkins, 2000; Finkler and Cullum, 2002). This result is also consistent with studies of reproduction-induced stress in other taxa. Reproductive females have higher stress levels than males in species of bats (measured via corticosterone, Klose et al., 2006), and birds (via heterophil:lymphocyte ratios; Kilgas et al., 2006). Interestingly, in our study, reproductive males did not have higher neutrophil:lymphocyte ratios than did nonreproductive individuals, suggesting that reproduction in male paedomorphic Mole Salamanders results in little to no increase in general physiological stress.

Besides the between-sex difference in neutrophil:lymphocyte ratios, we observed a higher variance in these ratios within female salamanders than in males (Fig. 1), indicating a greater variability in the stress response of females to reproduction. Indeed, the range of neutrophil:-lymphocyte ratios for females in this study was 0.17–1.53 (difference of 1.36), whereas for males, it was 0.05–0.64 (difference of 0.59). Natural variation in neutrophil (or heterophil):lymphocyte ratios certainly exists among animals (Ots et al., 1998; Davis et al., 2004), and our observation of variation within female salamanders may be an example of this. However, it is also possible that the female response to stress is related to the number or size of eggs she is carrying, which is known to vary among females (Semlitsch, 1985). If this is true, females carrying larger clutches may experience more stress than those carrying smaller clutches. By this same reasoning, the fact that females carry clutches of eggs internally during their reproductive state, whereas males carry the relatively lighter sperm, may be the driving factor in the overall sex-related differences in stress we observed.

Interestingly, nonreproductive individuals were larger than both reproductive males and females. However, the size of salamanders did not appear to influence neutrophil:lymphocyte ratios, contrary to our predictions. In other words, larger salamanders did not show higher levels of stress than smaller, based on our ANCOVA results and direct comparisons of size and neutrophil:lymphocyte ratios within males, females, and nonreproductive individuals. Because larger individuals invest more energy in reproduction (Semlitsch, 1985), we had expected to find size-related variation in neutrophil:lymphocyte ratios. However, Semlitsch (1985) did point out that the increase in reproductive effort by larger individuals he observed may be because energy availability also increases with increasing body size, which could explain why larger individuals in our study did not experience higher stress.

The leukocyte components of amphibian immune systems are increasingly being examined by field ecologists and herpetologists because of their potential to predict susceptibility to emerging diseases in amphibian populations (Forson and Storfer, 2006) and their utility in assessing the general health of individuals in stressful or polluted environments (Cabagna et al., 2005; Rutherford et al., 2005). The ratio of neutrophils to lymphocytes used here represents an important hematological parameter that has thus far only been used in laboratory studies in amphibians, but given its potential for assessing stress, we suggest it should be incorporated into future studies.

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