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ORIGINAL ARTICLE

# White blood cell profiles of breeding American toads (*Bufo americanus*) relative to sex and body size

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**Abstract** Immune traits may trade off against one another, or against other life history traits such as growth, development, and reproduction. Breeding introduces additional constraints on investment in immunity that may differ for each sex. During contests for access to females, males may be subjected to injuries that could result in infections. Thus, breeding males should show greater investment in first-line defenses against infection, as compared to other defenses, or as compared to investments by conspecific females. We tested these predictions of the immunoredistribution hypothesis by comparing white blood cell profiles of breeding male and female American toads. In this species, smaller males often occupy terrestrial positions as isolated satellites, while larger males are more likely to engage in attempted displacement of amplexed rivals, making the latter more likely to be injured and vulnerable to infections. Because heterophils are important first-line defenses against bacterial infections, we predicted that larger males would have higher proportions of heterophils in their leukocyte profiles than smaller males; this prediction was supported. However, contrary to expectation, females and larger males had similar proportions of heterophils, possibly because females were equally susceptible to injury during attempts to dislodge amplexed partners. Future work on white blood cell profiles of breeding amphibians is warranted, particularly for species where the sexes differ in likelihood of injury.

**Keywords** Immunity · American toad · Sexual selection · White blood cells

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## Introduction

Male vertebrates often are more likely to be parasitized than are conspecific females (e.g., Folstad et al. 1989; Poulin 1996; Schalk and Forbes 1997; Moore and Wilson 2002). That these differences can be most pronounced during the breeding season suggested that males have testosterone-mediated immunosuppression (IS; Folstad and Karter 1992). However, interactions between hormones and immunity are complex. Testosterone reduces certain components of humoral and cell-mediated immunity (Alexander and Stimson 1988), whereas estrogens appear to boost humoral immunity, but suppress cell-mediated immunity (Grossman 1985). Alternatively, corticosteroids may be the important immune suppressors in some species, whereas testosterone is simply a correlate of their activity (Evans et al. 2000). In response to stress, corticosteroid and estradiol-17β levels rise in blood of amphibians (Gobbetti and Zerani 1996). In other vertebrates, these hormones can cause shunting of leukocytes out of peripheral blood circulation and into the lymph nodes, skin, and immunological organs [immunoredistribution (IR)] where they are likely to detect antigenic challenges (Dhabhar and McEwen 1996; Dhabhar 1998). Thus, Braude et al. (1999) suggested that testosterone may induce or be associated with IR rather than IS. In contrast to IS, IR is thought to bolster aspects of immunity needed at particular times, such as breeding seasons. For example, male–male combat may lead to injuries; immune systems primed to respond to such injuries would make adaptive sense. As such, we may actually expect males to be more immunoresponsive than females to certain injuries such as skin lesions (Zuk and Johnsen 1998; Braude et al. 1999). IR also may explain other phenomena, such as why female birds are more likely to have blood parasites (McCurdy et al. 1998), because if male birds redistribute their immune cells to the skin, they may also be better equipped than females to fend off blood parasite infections that arrive via insect vectors.

In this paper, we examined white blood cell profiles of breeding American toads, *Bufo americanus*. To our knowledge, this is the first study that examines the IR hypothesis for amphibians, although several authors recognize IR as a viable alternative to IS (e.g., Adamo et al. 2001; Roulin et al. 2001; Adamo 2004). A basic premise of the IR hypothesis is that greater investment in one component of the immune system is thought to result in less investment in other components (such as production of certain cells or antibodies). Thus, IR may be confused easily with IS if only one or a few immune components are assayed (Braude et al. 1999). Furthermore, increased parasitism of males during the breeding season is expected (but see McCurdy et al. 1998), especially if the parasites chosen for study establish themselves because certain components of the immune system have been compromised in favor of others (Braude et al. 1999). More realistically, IR and IS likely cooccur. It is thus useful to determine investment in immune components because this might help explain differences in susceptibility to certain parasites and pathogens.

Hypotheses based on changes in immunity during breeding have the premise that immune responses can be costly (a premise with considerable support in vertebrates, particularly birds; Lochmiller and Deerenberg 2000). Breeding stresses can decrease ability of hosts of many taxa, including amphibians, to resist infection (Cooper et al. 1992; Gobbetti and Zerani 1996; Maniero and Carey 1997; Nordling et al. 1998; Oppliger et al. 1998). IS could take the form of reduced ability to produce leukocytes or their elements, thus resulting in a decrease in circulating leukocytes in the blood (Maule et al. 1988; Cooper et al. 1992; Maniero and Carey 1997; Hopster et al. 1998). However, circulating leukocytes also could decrease in blood if more of them are being diverted to skin, which would be IR (Braude et al. 1999). Braude et al. (1999) emphasized shunting of leukocytes to the skin, but IR could also occur in allocation. Differential white blood cell counts, a common technique in veterinarian laboratories, can help assess whether shunting, differential production, or both factors are occurring (different types of IR).

We compared white blood cell profiles of breeding male and female American toads. In Ohio, Illinois, and Ontario, the breeding season of American toads begins from late March-early April to late April and generally lasts 1 to 2 weeks (Licht 1976; Christein and Taylor 1978; Kruse and Mounce 1982; Fairchild 1984; Howard and Palmer 1995). The narrow window in which eggs are laid is referred to as explosive breeding (Sullivan 1992). Calling males usually arrive before, and tend to stay longer than, females at breeding ponds (Christein and Taylor 1978; Kruse and Mounce 1982). This can result in operational sex ratios of seven males per female in breeding ponds (Christein and Taylor 1978). This in turn means that males compete intensely for females (also see Arak 1983), risking injury and exposure to parasites and pathogens, as has been documented in salamanders (Bakkegard and Guyera 2004) and leopard frogs (McRuer DL, unpublished data). Amphibian leukocytes are similar to those of other vertebrates, consisting of eosinophils, basophils, monocytes, heterophils, and lymphocytes. Heterophils are homologous to neutrophils in higher vertebrates and serve as the first line of defense against internalized microbes (Black 1996; Horton and Ratcliffe 1998).

We tested whether males invest heavily in first-line defenses, namely heterophils. We also tested whether there were differences in heterophils in relation to size or condition of males. This second test is relevant for American toads because smaller males are often satellites that stay on land and try to intercept incoming females that are traveling to ponds to breed (Forester and Thompson 1998). The tactics of small males reduce the frequency with which they engage in interference competition with other males, making them less likely than larger males to sustain injuries. As such, heterophils may not be as necessary for small males. If large males have higher proportions of heterophils than females, and/or large males have higher proportions than small males, these results would support IR. Of course, size in toads is expected to correlate with age, and white blood cell profiles may relate more to age than to breeding and intrasexual competition. In this sense, females are a control for expectations of how white blood cell profiles vary with size in male toads. We did not make a specific prediction concerning size relations and immune profile in females, but examined these relations for comparison with similar relations in males.

## Materials and methods

Toads were sampled from Barb's Marsh at the Queen's University Biological Station in eastern Ontario, Canada (45° 34′ N and 76° 13′ W). We wore latex gloves and captured toads by hand. We used a new pair of gloves for each toad to prevent transfer of pathogens. Approximately 30 s after capture, 0.02–0.04 ml of ventricular blood was obtained by cardiac puncture using a 28-gauge (0.5 cc) needle. The toad was then placed upside-down on a 12×20-cm cardboard card and the length between the snout and vent (SVL) was recorded. The measurement was repeated on the other side of the card (one card per specimen). We also weighed toads in a preweighed bag using 100-g Pesola spring scales. The first (outer) digit on the hind left foot was clipped using a pair of human nail clippers stored in 70% methanol to indicate that the animal had been sampled. The toad was then released on site. All procedures for handling and sampling toads were done in accordance with guidelines of the Canadian Council on Animal Care (1984). In total, 27 toads were processed.

For differential blood cell counts, several drops of blood obtained by cardiac puncture were used to make a blood smear, following Bennett (1970). Slides were air-dried and later stained. Staining and counting of blood cells was done by Vita-Tech, Mississauga, Ontario. Cells that were subject to drying artifact, which makes cytoplasmic and nuclear details unrecognizable, were not included in the differential leukocyte count (in fact, fields of view were considered only if all leukocytes could be identified). For standard differential counts, 100 leukocytes were classified per smear.

Analyses were conducted using SAS software (SAS Institute Inc. 2000). We report means±SE. Confidence intervals for estimates of proportions of heterophils were calculated using the Clopper–Pearson method (Zar 1996).

## Results

As for other populations of American toads, females at Barb's Marsh were heavier than males (95.4±4.3 g vs 60.0±4.1 g, respectively; $t$=5.9, $df$=25, $P$<0.0001). Females also had longer snout-vent lengths (SVLs) than males (89.2±1.7 mm vs 77.4±1.7 mm, respectively; $t$=4.9, $df$=25, $P$<0.001). For tests relating either mass or SVL to proportions of white blood cells, we performed analyses separately by sex, because males and females differed significantly in these variables. ANCOVA models could not be used because the covariate (SVL) was dependent on the class variable (sex).

We were interested in two predictions relating to IR. First, we tested whether the sexes differed in proportion of any white blood cells and then whether they differed specifically in heterophils. Over all individuals, proportion of heterophils averaged 68±2.0%, lymphocytes 20±1.7%, monocytes 1.5±0.4%, eosinophils 3.3±0.6%, and basophils 7.4±1.0%. Thus, lymphocytes and heterophils together accounted for close to 90% of white blood cells. Females and males differed very little in proportions of their white blood cells, ranging from a difference of 0.6% for eosinophils to 4.0% for heterophils. We found no sex differences in blood cell profiles (MANOVA, Wilk's Lambda=0.87, $F_{5,21}$=0.6, $P$=0.68). In contrast to our prediction, there also were no differences between sexes in proportions of heterophils (males 66±2.7%, females 70±2.9%; $t$=0.9, $df$=25, $P$=0.38).

Consistent with prediction, for the 14 males, there was a significant, positive, linear, relation between SVL and the proportion of heterophils (Fig. 1), described by the following equation: percent of heterophils (hereafter $H$)=−36.1+1.32 SVL ($R^2$=0.31, $P$=0.04). For 13 females, the relation was not significant: $H$=−57.9+0.13 SVL ($R^2$=0.01, $P$=0.73; Fig. 1). Perhaps surprisingly, the trend of mass increasing with SVL was not significant for either sex (females: mass −15.2+1.2 SVL; $R^2$=0.22, $F$=3.1, $P$=0.10; males: mass −3.2+0.82 SVL, $R^2$=0.15, $F$=2.1, $P$=0.17).

We next tested whether condition related to proportions of heterophils for females and males, where condition was defined as the residual mass from the above relations between mass and SVL for each sex evaluated separately. For females, there was a near significant trend for the proportion of heterophils to decline with residual mass (hereafter RM) ($H$=69.6–0.2 RM, $R^2$=0.30, $F$=4.5, $P$=0.056) whereas the relationship was not significant in males ($H$=66–0.18 RM, $R^2$=0.02, $F$=0.3, $P$=0.61).

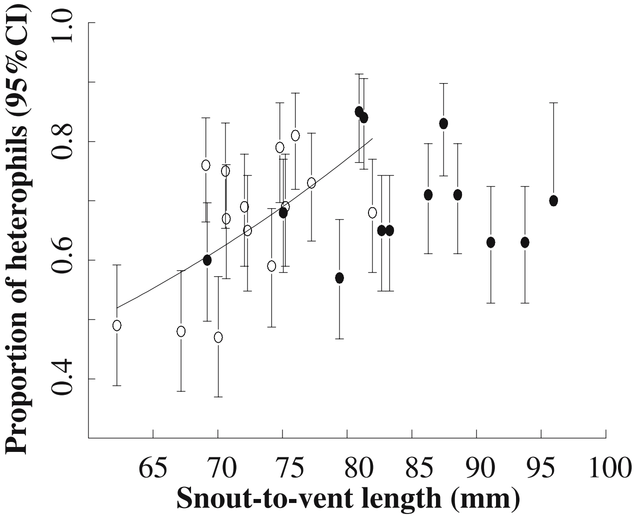

**Fig. 1** Larger male American toads had a significantly higher proportion of heterophils in their leukocytes, whereas there was no significant relationship between female body size and their heterophil proportion. *Open circles* are males, *filled circles* are females, and *line* is least squares regression line relating proportion of heterophils to snout to vent lengths for males only. Confidence intervals are calculated using the Clopper–Pearson method (Zar 1996)

## Discussion

Our results show that the sexes differed in the relations between first-line defenses (proportion of heterophils) and body size, and support IR within males. However, we initially predicted the sexes would differ in proportion of heterophils independent of such relations. This lack of sex differences in white blood cell profiles is interesting. As we noted, breeding males are not a homogeneous group; small males were not expected to engage in male–male competition that leads to injury. Their inclusion in this study means that average heterophil counts for males are reduced because of the relation between proportion of heterophils and body size in males. Yet females, independent of their body size, achieved heterophil counts on par with large males. Small females did not have reduced heterophil counts as would be expected if age was a determinant of this aspect of immunity.

Another aspect of this species' natural history may help explain the absence of sex biases in proportions of heterophils. All females that are receptive are likely amplexed, whereas not all males are amplexed (Howard 1988). Furthermore, at our study site, one to two other males can attempt to displace the amplexed male and cause injury to the female. It is quite likely that females can sustain injuries and even drown (the only dead toads found at our study area were both females). Thus, females may be frequently injured and would be expected to invest in first-line defenses. It would be useful to repeat this study on species where the sexes differ in rates of injury. Unfortunately, we could not measure injuries in this species easily, but found that males more often had skin lesions than did females in another explosive breeder, *Rana pipiens* (McRuer DL, unpublished data).

It is noteworthy that females showed no relation between size and proportion of heterophils; however, they did tend to have reduced heterophil counts when in relatively good condition. We suspect that females in good condition may have been less stressed than other females, and stress may influence immune parameters. In another study, juvenile leopard frogs held in bait shops under stressful conditions had reduced condition based on liver mass, but higher heterophil counts than wild leopard frogs of the same size and age (McRuer 2001).

Our results are most consistent with the IR hypothesis principally as it relates to between-male variation in breeding tactics and its relation to intraspecific contact or combat, and exposure. Future studies should examine metrics of spleen size or weights between the sexes of amphibians, as has been done for birds (Møller et al. 1998; Shutler et al. 1999; Brown and Bomberger Brown 2002). The spleen acts immunologically as the primary site of interaction between the body's leukocytes and invading pathogens (Horton and Ratcliffe 1998). Therefore, an enlarged spleen could be the result or either a heightened immune response, a current infection, or a combination of these. Reallocating specific leukocytes to the spleen would reduce their proportions in circulation, consistent with a logistical consideration in testing the IR hypothesis (Braude et al. 1999).

Recently, there have been many studies using standard veterinarian techniques to examine white blood cell profiles of vertebrates to address several questions about the ecology and evolution of immunity (e.g., Pap and Márkus 2003; Shutler et al. 2004; Edwards et al. 2006). Our results add to this general area of investigation. We recognize that cardiac blood may not be representative of differential leukocyte densities near the skin (returning to the point about redistribution vs reallocation). However, we obtained results more consistent with general reallocation, which may be expected to result in both enlarged spleens, and higher leukocyte densities near the skin. The fact that we obtained strong relations with small samples suggests that that future studies on white blood cell profiles of breeding amphibians are warranted, particularly for species where injury rates differ by sex.

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