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The effect of long-term corticosterone treatment on blood cell differentials and function in laboratory and wild-caught amphibian models

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ABSTRACT

The effect of long-term stress on amphibian immunity is not well understood. We modeled a long-term endocrine stress scenario by elevating plasma corticosterone in two species of amphibians and examined effects on white blood cell differentials and innate immune activity. Plasma corticosterone was elevated in American bullfrogs (*Lithobates catesbeianus*) by surgically implanting corticosterone capsules and in African clawed frogs (*Xenopus laevis*) by immersion in corticosterone-treated water. To provide a context for our results within endogenous corticosterone fluctuations, diurnal plasma corticosterone cycles were determined. A daily low of corticosterone was observed in *X. laevis* at 12:00, while a significant pattern was not observed in *L. catesbeianus*. Elevated plasma corticosterone levels increased the ratio of peripheral neutrophils to lymphocytes, in both species, and decreased eosinophil concentrations in *L. catesbeianus* over a long-term period. Whole blood oxidative burst generally correlated with neutrophil concentrations, and thus was increased with corticosterone treatment, significantly in *L. catesbeianus*. In *L. catesbeianus*, an endogenous response of eosinophils and lymphocytes to implanted empty (sham) capsules was observed, but this effect was attenuated by corticosterone. Peripheral monocyte and basophil concentrations were not significantly altered by corticosterone treatment in either species. Our results show that long-term stress can alter amphibian immune parameters for extended periods and may play a role in susceptibility to disease.

1. Introduction

Increased glucocorticoid hormone secretion is a central action of the vertebrate response to stressful conditions, real or perceived. In addition to the primary role of glucocorticoids in metabolism, the effect of glucocorticoid hormones on immunity and inflammation is well established (Chrousos, 1995; Sapolsky et al., 2000). However, stress likely influences immune function through many mechanisms and may result in a spectrum of effects (Sapolsky et al., 2000). Indeed, reports of the effects of stress on immunity in wildlife highlight the importance of a diverse range of factors such as magnitude of stressor, duration, and the life stages affected on the ultimate result of the stress response in an individual (reviewed in Martin, 2009). The current understanding of the mechanisms by which long-term stress influences vertebrate immune systems is incomplete. In addition, the results of such study have direct and immediate application to wildlife in many habitats, as human-caused environmental change represents a challenge to wildlife that is likely to persist for extended periods of time (Vitousek et al., 1997). In the current report, we examine long-term effects of elevated glucocorticoids on the immune system in amphibians, a vertebrate class that is highly threatened by environmental change and disease challenges (Stuart et al., 2004). We also provide further evidence that anuran amphibians respond similarly to long-term stress by including results from two species.

Corticosterone (CORT) is the primary glucocorticoid secreted by the interrenals in anuran amphibians (Jungreis et al., 1970). CORT levels fluctuate in postmetamorphic amphibians with season, breeding activity, and environmental conditions (Dupont et al., 1979; Licht et al., 1983; Mendonca et al., 1985; Pancak and Taylor, 1983; Thurmond et al., 1986). Environmental regulation of endocrine responses allows amphibians to adapt to dynamic aquatic ecosystems; however, the influence of environmental signals on physiology may leave amphibians particularly vulnerable

Abbreviation: CORT, corticosterone.

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to endocrine disruption in altered habitats. Several reports suggest that amphibians in altered habitat conditions have increased plasma CORT and interrenal dysfunction (Gendron et al., 1997; Hopkins et al., 1997, 1999). Increased plasma CORT was also observed in laboratory studies of agrochemical exposure (Hayes et al., 2006; McMahon et al., 2011). The degree to which long-term environmental stressors, both natural and human caused, affect immune function in amphibians is not well understood.

Previous studies of the effects of glucocorticoids on amphibian immune function provide clear evidence that amphibians are cortico-sensitive but have (primarily) been limited to short-term exposures (hours to days) with single collection time points. Several experiments show directly that short-term glucocorticoid and corticotrophin treatments result in increased peripheral neutrophils and decreased peripheral lymphocytes (Bennett et al., 1972; Bennett and Johnson, 1973). Blood cell differential changes are a common response of many vertebrates to stress, and likely controlled by redistribution of leukocytes between the blood and immune tissues (Davis et al., 2008; Dhabhar et al., 1995). In addition to changes in the peripheral blood cell differentials, glucocorticoid treatment of adult amphibians caused involution and decreased cellularity of the thymus and spleen (Ducoroy et al., 1999; Tournefier, 1982) and decreased antigen binding (Ruben and Vaughan, 1974), but did not alter allograft rejection response (Tournefier, 1982). The magnitude of corticoid exposure and timing of antigen challenge may greatly influence immune response, however. For example, corticoid exposure stimulated antigen binding in one study of axolotls (Tournefier, 1982). In larvae treated with CORT for longer periods from hatching through metamorphosis, skin gland development was inhibited (Hayes and Gill, 1995), peripheral eosinophil concentrations were decreased (Belden and Kiesecker, 2005) and involution of the thymus and spleen were observed (Hayes, 1995). The synthetic corticosteroid, dexamethasone, decreased peripheral lymphocytes (Garrido et al., 1987), altered the cellularity of the spleen (Garrido et al., 1987), caused involution of the thymus (Garrido et al., 1987), and decreased the phagocytic activity of peritoneal neutrophils in one long-term study (Froese et al., 2005).

No single model amphibian species has been studied consistently and the wide range of amphibian species limits the ability to draw comparative conclusions. Though declining amphibian populations further emphasize the importance of knowledge-based management, experiments in threatened and declining amphibian populations are often limited by sample size or nonlethal methods. Therefore, it is important to establish model systems in which results can be used to direct more specific experiments in sensitive populations. This study employs two commonly available species in North America: the African clawed frog (*Xenopus laevis*) and the American bullfrog (*Lithobates catesbeianus*). *X. laevis* are readily available from commercial supply houses and are relatively easy to maintain in captivity. *X. laevis* are frequently used as model organisms in many experiments on amphibians. However, differences in physiology exist between *X. laevis* and other anurans (Duellman and Trueb, 1986). It is crucial to understand these differences in order to best model experiments intended to provide information for free ranging amphibians. We provided a comparison to *X. laevis* using *L. catesbeianus*. *L. catesbeianus* are easily collected from many localities in the United States and share similar life history to many North American anurans from temperate regions. Therefore studies of the influence of environmental conditions or laboratory treatments on *L. catesbeianus* may provide valuable information for conservation of declining species.

Given the evidence that the immune system of amphibians is cortico-sensitive for short time periods, the need for further information on the effects of long-term stress (days to weeks) is highlighted. Amphibians regularly encounter pathogens in both wild and captive settings, and disease is a primary driver of global declines in amphibian populations inhabiting both altered and seemingly pristine environments (Stuart et al., 2004). Here we report the effects of long-term elevated CORT on two common measures of amphibian immunity, blood cell differentials and whole blood oxidative burst activity in two species of amphibian.

2. Material and methods

2.1. Experiment 1 – the effect of CORT implants on American bullfrog (*L. catesbeianus*) blood cell differentials and blood cell activity

2.1.1. *L. catesbeianus*

Adult American bullfrogs (*L. catesbeianus*) were collected from Pleasanton Ridge East Bay Regional Park, Pleasanton, California, USA (37 38′43.56″N 121 55′06.01″W) where there is no current or recent agricultural activity, between July and November 2008. At the size collected, the sex of *L. catesbeianus* cannot be determined without dissection. Because of known hormone fluctuations in adult male *L. catesbeianus*, we limited experimental animals to individuals that did not display signs of male sexual maturity and secondary sex characteristics (yellow throat coloration, enlarged tympanum:eye ratio, enlarged thumb breeding glands) (Licht et al., 1983; Mendonca et al., 1985). For this reason, our experiment included both immature male and female animals. Though we were unable to verify the exact age of wild-caught animals, the body size of our experimental animals at the time of collection indicates an age of at least one year post metamorphosis. Anurans of this age should have an adult type immune system of relevance to our study (Rollins-Smith, 1998). Animals were group-housed in the laboratory for several months prior to the start of experiments and fed Ca_2CO_3 dusted 5-week old crickets *ad libitum* every three days. Prior to starting the experiment, no animals displayed symptoms of poor health.

2.1.2. Experimental conditions

Animals were apportioned to treatment groups such that there were no significant differences in body weight or length between treatments (see Section 3.1.1). *L. catesbeianus* used in this study were 128.7 ± 32.6 g and 11.4 ± 1.16 cm (snout-vent length). For the duration of all experimental exposures, animals were housed individually in clear polyethylene tanks (259 mm W × 476 mm L × 209 mm H; Allentown, N.J.). After moving animals from group housing to individual tanks, animals were acclimated to individual housing for two water changes prior to beginning of treatment. Tanks were elevated approximately 5 cm on one end and provided with 1 L of 0.1 × Holtfreter's solution (6.03 mM NaCl, 0.068 mM KCl, 0.09 mM $CaCl_2$, and 0.24 mM $NaCO_3$; Holtfreter, 1931). Water in our animal facility is UV-treated, carbon filtered, with no detectable pesticide residues. Every third day the Holtfreter's solution was removed, tanks were cleaned, and new Holtfreter's solution was added. Paper towels (unbleached) were placed in each tank to provide a perch to simulate a semi-terrestrial habitat. White styrofoam dividers were placed between all tanks to ensure that animals in adjacent tanks could not view each other. The experiment was conducted at 22 (±1) °C and lights were operated on a 12 h light:12 h dark cycle, lights on at 07:00. Animals were fed eight 5-week old crickets dusted with Ca_2CO_3 on the day before each water renewal. Tanks were systematically rotated on the day of water renewal to avoid positional effects throughout the experiment. Implants and subsequent analysis were conducted blindly using color codes for each treatment.

2.1.3. CORT capsules

Plasma CORT was elevated in *L. catesbeianus* by surgically implanting CORT-filled (purity > 98.5%; Sigma Aldrich; St. Louis,