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Corticosterone–immune interactions during captive stress in invading Australian cane toads (*Rhinella marina*)

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ABSTRACT

Vertebrates cope with physiological challenges using two major mechanisms: the immune system and the hypothalamic pituitary–adrenal axis (*e.g.*, the glucocorticoid stress response). Because the two systems are tightly integrated, we need simultaneous studies of both systems, in a range of species, to understand how vertebrates respond to novel challenges. To clarify how glucocorticoids modulate the amphibian immune system, we measured three immune parameters and plasma corticosterone (CORT), before and after inflicting a stressor (capture and captive confinement) on introduced cane toads (*Rhinella marina*) near their invasion front in Australia. Stress increased CORT levels, decreased complement lysis capacity, increased leukocyte oxidative burst, and did not change heterologous erythrocyte agglutination. The strength of the CORT response was positively correlated with leukocyte oxidative burst, and morphological features associated with invasiveness in cane toads (relative leg length) were correlated with stress responsiveness. No immune parameter that we measured was affected by a toad's infection by a parasitic nematode (*Rhabdias pseudosphaerocephala*), but the CORT response was muted in infected *versus* uninfected toads. These results illustrate the complex immune–stress interactions in wild populations of a non-traditional model vertebrate species, and describe immune adaptations of an important invasive species.

Introduction

Anthropogenic stressors (*e.g.*, habitat loss, climate change, pollution, invasive species) represent unprecedented threats to wild vertebrate populations, and have the capacity to simultaneously challenge two body systems that are often employed to cope with such stressors: the hypothalamic pituitary–adrenal (HPA) axis, and the immune system (Martin et al., 2010). These effects are often due to the indirect effects of these stressors on the HPA axis – the hormone cascade that modulates the stress response – and its effects on the immune system; there is evidence from diverse vertebrate fauna that the HPA axis affects various immune parameters (Martin, 2009; Sapolsky et al., 2000). Therefore, studies that simultaneously measure components of both the immune system and the HPA axis are needed to determine the physiological and functional consequences of these new challenges.

Historically, interactions between the HPA axis and the immune system were thought to be negative and immunosuppressive (Sapolsky et al., 2000); however, modern views support a more nuanced explanation. Currently, the HPA axis is thought to modulate the immune response in two important, and not necessarily negative, ways: (1) immunomodulation during the stress response contributes to the control of a potentially lethal and inappropriate post-stressor immune response (Sapolsky et al., 2000), and (2) immunomodulation by immune "trafficking", whereby immune agents are shifted from circulation to sites of need (*e.g.*, skin), and thus appear to be undergoing suppression (Dhabhar and McEwen, 1997, 1999). These hypotheses, and observations of ecological trade-offs between immunity and other life history components (*e.g.*, reproduction, growth: French et al., 2007, 2009), predict that nonspecific, innate immune factors are often the target of immunomodulation by the HPA axis (Lee and Klasing, 2004; Norris and Evans, 2000); these components of innate immunity are prepared to respond immediately to infectious agents but also contribute to non-specific inflammatory responses that are potentially harmful and/or energetically expensive to the host.

This general depiction of stress–immune interaction was largely developed from studies in mammals (Sapolsky et al., 2000), and additional studies in diverse vertebrates will likely contribute to our understanding of these interactions (*e.g.*, Somero, 2000). Studies have demonstrated immunological changes associated with various challenges in amphibians, including metamorphosis (Rollins-Smith, 1998), reproduction (McCallum and Trauth, 2007), and experimental administration of corticosteroids (Bennett et al., 1972; Rollins-Smith et al., 1997). Hematological changes during captive stress have also been documented in amphibians (Davis et al., 2008; Rollins-Smith,

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2001); however, multiple species comparisons and additional measures of immunity are needed to identify any ecological or evolutionary patterns in these trends (Demas et al., 2011). In addition, such information may aid our understanding of global amphibian declines, which are hypothesized to be the result of disease and synergistic effects of multiple anthropogenic stressors (*e.g.*, pollutants, habitat modification and species introductions) influencing amphibian immunity, reproduction, and survival (Hayes et al., 2010).

The cane toad, *Rhinella marina* (formerly *Bufo marinus*), is an excellent study species to investigate immune–endocrine interactions in amphibians, for both practical and exploratory reasons. Their status as a high profile invasive species in Australia has made them a model system for the study of ecological invasion dynamics and evolution (see Shine, 2012), and therefore, hypotheses regarding the immune capacity of invasive vertebrates (*e.g.*, Lee and Klasing, 2004) can also be rigorously tested using this study species. Finally, more is known about immune function in *R. marina* than perhaps all other amphibian model species except *Xenopus laevis*, allowing new immunological data to be placed in a robust context (*e.g.*, Cone and Marchalonis, 1972; Diener and Nossal, 1966; Lin et al., 1971; Lin and Rowlands, 1973; Weinheimer et al., 1971). A previous study demonstrated a link between the features that facilitate rapid dispersal (*e.g.*, greater relative tibia length, longer legs, faster, more linear movement), in invading toads and a potentially stress-induced spinal condition (Brown et al., 2007), supporting the hypothesis that additional ecologically important associations between immunity and stress will manifest in these toads. Additionally, cane toads in their invasive Australian range are commonly infected with a nematode lungworm (*Rhabdias pseudosphaerocephala*) native to the toad's ancestral range in South America (Dubey and Shine, 2008). Field studies reveal that this nematode reduces the growth rates of adult cane toads (Kelehear et al., 2011) and laboratory infections in metamorph cane toads caused reduced growth rates, locomotor performance, and often death (Kelehear et al., 2009). This parasite is common in our study area with a mean prevalence (the percentage of potential hosts that possess parasites) of 61%, and these infections can comprise many individual lungworms, with infection intensity (the number of parasites per infected host) as high as 98 worms (Kelehear et al., 2011). Considering the pathogenicity and prevalence of this parasite, we expect that infection status will influence the outcome of immune–endocrine interactions.

To determine the extent to which stressors can modulate the immune profile of toads, we tested the following predictions in wild-caught cane toads near their current invasion front in Australia: (1) captive stress increases levels of a steroid stress hormone (corticosterone [CORT]); (2) captive stress affects immune parameters; (3) consistent with the hypothesis of immune modulation by the HPA axis, levels of plasma CORT will correlate with immune parameters; and (4) factors associated with invasiveness (*e.g.*, relative tibia length; Brown et al., 2007; Llewellyn et al., 2012) will be associated with increased baseline CORT and decreased immune function. Finally, (5) we considered whether the infection prevalence and intensity of a common nematode lungworm (*R. pseudosphaerocepha*la) is associated with immunological parameters or the CORT response. Three measures of immunity assessed by functional immune assays (whole blood phagocytosis, plasma hemagglutination, and plasma bacterial killing capacity) were utilized as a broad measure of immune capacity in these toads (*e.g.*, Demas et al., 2011).

Materials and methods

Blood sampling and processing

We captured 55 toads by hand at night (1930–2230 h; $n=30$ collected 9 February 2011, $n=25$ collected 6 March 2011) on local roads near the University of Sydney Tropical Ecology Research Facility at Middle Point, Northern Territory, Australia (12°37′S, 131°18′E). We attempted to collect sex ratios evenly. We drew 800 μL of blood within 3 min of capture *via* cardiac puncture (with a sodium-heparinized 25 G × 16 mm needle; 1 mL Terumo® syringe) for all toads except a blood-loss control group of 10 toads collected on the latter date. We handled the blood-loss control toads in an identical manner, including insertion of a sodium-heparinized needle into the heart, except we drew no blood from the heart. These blood-loss control animals served to investigate whether any observed stress effects may be attributed to blood loss alone.

From the 800 μL sample, we aliquoted the blood into two vials: one vial (350 μL) of whole blood was refrigerated overnight, and the other was centrifuged immediately using a battery-powered field centrifuge. We then drew off the plasma and froze it immediately in a car battery-powered freezer (−20 °C; Waeco Coolfreeze CF-40), transported it back to the Research Facility that night, and transferred into another −20 °C freezer. We assessed whole blood phagocytosis the next morning from the refrigerated whole blood (sample refrigeration time: 9.5–12.5 h; phagocytic capacity of refrigerated amphibian blood remains stable for at least 2 days post-collection: Papp and Smits, 2007), and assessed plasma hemagglutination and bacterial lysis from thawed plasma approximately 20 h post-collection (plasma can be frozen for up to two weeks and maintain its complement viability: Sherwood, 1951). Leftover plasma was refrozen and stored at −20 °C for CORT analysis.

Captive stress protocol

After we took the initial blood sample, we placed the toads into individual, labeled cloth bags (approximately 4 L volume), and placed these gently into an air-conditioned vehicle and transported them immediately to the Research Facility. We moistened the bags and placed them into buckets (approximately 8 bags per bucket) for the night. Next morning (0830–1130 h), we bled all the toads again (as above), bled the 10 blood-loss control toads for the first time, and processed blood samples as above. We ran assays again on the captive stress-treated toads after an equal amount of time between bleed and assay as in the baseline treatment. Levels of circulating CORT can vary with time of day. Since we collected our baseline samples at night and our stressed samples during the day, our data is potentially confounded by the time of collection. However, CORT often increases in response to increased metabolic and energetic demands; therefore, peak CORT levels generally coincide with peak activity levels. For instance, CORT peaked at dusk (1730 h) and was lowest in the morning (0930 h) in the nocturnal toad, *Anaxyrus* (=*Bufo*) *americanus* (Pancak and Taylor, 1983), and in diurnal reptiles, corticosterone was lowest at night (2100 h) in the skink, *Egernia whitii* (Jones and Bell, 2004), and the iguana, *Amblyrhynchus cristatus* (Woodley et al., 2003). There was no influence of time of day on CORT levels in the tuatara, *Sphenodon punctatus* (Tyrrell and Cree, 1998) or the hellbender, *Cryptobranchus alleganiensis* (Hopkins and DuRant, 2011). If CORT does follow a diel cycle in nocturnal *R. marina*, we would predict CORT to be lowest in the morning, and therefore we do not think our sampling regime will confound our results.

Plasma corticosterone

On 29 August 2011 (175 and 200 days post-collection), we thawed plasma samples (baseline $n=38$, and stressed $n=40$) and assessed their CORT concentration using a commercial enzyme immunoassay kit (Corticosterone HS EIA; ImmunoDiagnostic Systems Ltd., Bolden). Kits can accurately measure CORT concentrations between 0.3 and 20 ng/mL. We diluted plasma 1/5 with assay buffer (20 μL plasma + 80 μL buffer) so that samples fell within the detectable range of the assay's standard curve (dilution ascertained by a previous study in our lab: Brown et al., 2011).