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Differential gene expression to an LPS challenge in relation to exogenous corticosterone in the invasive cane toad (*Rhinella marina*)

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ABSTRACT

The cane toad (*Rhinella marina*) is an invasive amphibian in several parts of the world. Much of the research performed on assessing the dispersal potential of invasive species has focused immunity. Invaders are predicted to rely less on pro-inflammatory immunity, allowing them to allocate energy to dispersal. Elevated stress may play a role in regulation of immune responses used by invasive species. RNA sequencing of spleen tissue from cane toads subjected to an acute LPS challenge revealed genes coding for cytokines involved in typical innate responses such as phagocytic cell recruitment, extravasation, inflammation, and lymphocyte differentiation were significantly upregulated, while toads receiving transdermal application of corticosterone in addition to an LPS injection showed downregulation of genes involved with cell mediated immunity. These results indicate hormonal changes associated with acute stress may alter investment into mounting cell-mediated or humoral responses while allowing for prolonged phagocytic innate responses in this invasive species.

1. Introduction

Diversity of many taxa around the world is declining (Light and Marchetti, 2007; Pimm et al., 1995; Stuart et al., 2004). Among the list of potential variables that lead to species diversity declines are invasive species (Wilcove et al., 1998), which have been shown to negatively affect native fauna through various interactions including spreading disease (Nally et al., 2016), outcompeting and preying upon native species (Willson, 2017), and hybridizing with native species (Mooney and Cleland, 2001). Researchers assessing physiological reasons behind the establishment and spread of invasive species have utilized RNA sequencing (RNA-seq) to generate novel transcriptomes to assess of gene expression of various physiological processes in invaders that may contribute to their success (Akbari et al., 2013; Ioannidis et al., 2014; Manfrin et al., 2015), as many of these species lack sequenced genomes (Ekblom and Galindo, 2011; Hornett and Wheat, 2012).

The Evolution of Increased Competitive Ability (EICA) hypothesis (Lee and Klasing, 2004), predicts that as non-native species escape pathogens from their native range when introduced into a new environment, they allocate less energy to immune responses and more to functions that facilitate dispersal, contributing to further spread into the introduced habitat (Torchin et al., 2003). As these non-native species did not co-evolve with pathogens present in the new habitat, pathogenic risk may be reduced as many novel pathogens encountered are not harmful (Torchin et al., 2003), while excessive immune responses may lead to autoimmune complications (Hanssen et al., 2004). Aggravated innate immune responses may also lead to systemic inflammation, an energetically costly response affecting overall growth and development (Spurlock, 1997), which is sometimes fatal (Lee and Klasing, 2004). In mammals, certain components of adaptive immunity, such as cell mediated responses, may also lead to inflammation (Halloran et al., 2016) which could reduce dispersal further into the range. Therefore, rapidly invading species are predicted by the EICA hypothesis to rely more on humoral antibody responses, avoiding inflammatory immune responses, while still retaining the ability to phagocytize pathogens (Lee and Klasing, 2004).

In addition to their escape from native pathogens, stress responses may also play a role in differential immune responses of invasive species (Raberg et al., 1998). As organisms perceive danger or experience stressful environmental conditions, glucocorticoids are released from the adrenal glands, interrenal glands in amphibians, with the main glucocorticoid produced in amphibians being corticosterone (Rollins-

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Smith, 2017) following hormonal signals from the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis (Kloet et al., 2008). These compounds have varying physiological effects, including mobilizing energy reserves while altering cardiovascular function, as well as altering feeding and reproductive behavior (Johnson et al., 1992), all of which serve to allocate resources to allow the organism to cope with the perceived threat or situation. Although beneficial in some cases, the allocation of energy to stress responses can come at a cost to other physiological processes such as immune responses, which may have differing energetic costs (Lee and Klasing, 2004). In addition to the stressful conditions an invading species may encounter, elevated stress is also correlated with dispersal (Belthoff and Dufty Jr. 1998; Landys et al., 2004). Chronically elevated corticosterone has been shown to suppress adaptive immune responses such as humoral and cell-mediated immunity in birds (Bourgeon and Raclot, 2006; Stier et al., 2009; Saino et al., 2003), and be correlated with increased susceptibility to infection in amphibians (Rollins-Smith, 2001). In cane toads, Graham et al. (2012) documented that phagocytic activity increases while complement activity decreases with acute elevated corticosterone levels.

Injection of lipopolysaccharide (LPS), a pathogen-associated molecular pattern (PAMP) of gram-negative bacteria (Mogensen, 2009), has been used to challenge and assess immune responses of multiple organisms, including invasive species (Lee et al., 2006; Llewellyn et al., 2012). LPS activates the toll-like receptor 4 (TLR4) signaling pathway in macrophages, neutrophils, and dendritic cells of the innate immune systems of mammals (Hemmi and Akira, 2005; Molteni et al., 2016; Sabroe et al., 2003). Existence of a homologue of TLR4 has been shown in amphibians as well (Fitzgerald et al., 2001; Ishii et al., 2007). In mammals, this activation leads to a signal transduction pathway resulting in the transcription factor NF-kB being translocated to the nucleus (Lu et al., 2008), inducing expression of various chemical messengers and cytokines characteristic of innate and adaptive immune responses (Lawrence, 2009). It has been suggested that glucocorticoids interfere with NF-kB activity, and could explain the differing immune responses following elevated stress (Padgett and Glaser, 2003).

Cane toads (*Rhinella marina*) are among the world's largest amphibians (Lever, 2001), and are native to regions of central and South America (Zug and Zug, 1979). Due to their voracious appetites, these toads have been introduced to several regions throughout the world as a means of controlling for various invertebrate pests (Lever, 2001), including Florida in the 1930's (Hero and Stoneham, 2005). Although there are reports of cane toads being successful in this regard, (Chen and Kovar-Kova, 1967), in many cases the toads failed and instead dispersed rapidly (Shine, 2010). These toads have been shown to reach dispersal rates of up to 55 km yr^{-1} in Australia (Phillips et al., 2007), with female toads laying up to 30,000 eggs per clutch in invaded areas (Hagman and Shine, 2006). As the habitat range of this invasive toad expands, competition with native species may occur (Shine, 2014); however, as these toads are toxic throughout their lifecycle, the main detrimental effect to native wildlife from exposure is poisoning of predators (Shine, 2010). Although when compared with mammals, amphibians are more resistant to LPS (Berczi et al., 1966), multiple studies using LPS to stimulate the immune systems of amphibians have been performed (Sherman and Stephens, 1998; Zou et al., 2000; Cui et al., 2011; Llewellyn et al., 2012; Goetz et al., 2017; Selechnik et al., 2017; Iglesias-Carrasco et al., 2018), and previous research has shown that cane toads on the invasion front in Australia have differing responses to LPS compared with toads from longer-established populations (Llewellyn et al., 2012), although the molecular mechanisms explaining the differing responses remain unclear (Brown et al., 2018).

Characterizing the differentially expressed genes in invasive cane toads responding to an immune challenge with LPS and assessing how expression is affected by elevated corticosterone levels would be useful in future work further assessing invasive potential of toads on the invasion front and comparing these responses across longer established populations. Therefore, the objectives of this research were (1) to utilize a transcriptomic approach to obtain novel primers for pro-inflammatory immune genes in the invasive cane toad, and (2) to assess differential gene expression following an acute immune or immune and stress challenge with either LPS only or LPS and corticosterone, respectively. Our hypothesis was that many immune genes upregulated during the challenge with LPS would be downregulated when corticosterone was administered simultaneously, indicating cost-tradeoffs between mounting an immune response and allocating energy to the fight-or-flight response.

2. Materials and methods

2.1. Innate immune and stress challenge

A total of twenty-seven adult toads with similar body masses (~105 g) and snout-vent-lengths (~14 cm) collected under permit EXOT-17-34 from New Port Richey FL, a population near the northern invasion-front in Florida, were divided into three treatment groups (n = 9). These three groups, comprised of 6 male and 3 female toads each, consisted of the following: 1) Control: a treatment group receiving 5 μL of peanut oil 1 h before and 1 h after an intraperitoneal injection of sterile PBS (2 μL of PBS/g of body mass), 2) LPS: a treatment group receiving 5 μL of peanut oil 1 h before and 1 h after an intraperitoneal injection of LPS (2 μL of LPS solution/g of body mass, a dose that has been used to elicit decreased rates of activity, feeding, and dispersal (Llewellyn et al., 2010; Brown et al., 2018) as well as increased metabolic responses in this species (Llewellyn et al., 2012)), and 3) LPS with corticosterone: a treatment group receiving 5 μL of a corticosterone solution 1 h before and 1 h after an intraperitoneal injection of LPS (2 μL of LPS solution/g of body mass). The LPS stock solution was made by diluting 1 mg of LPS (Sigma Aldrich O55:B5) in 1 mL of sterile PBS, while the corticosterone stock solution of 3 mg/mL was made by dissolving powdered corticosterone (Sigma Aldrich 2505) in peanut oil. Each animal received two 5 μL drops of corticosterone solution (15 μg of corticosterone $drop^{-1}$ for 30 μg total), at the dorsal region, between the front legs (or the "neck region"), similar to the methods used by Assis et al. (2015). Two drops of the corticosterone solution (or peanut oil only in the case of the control and LPS treatment groups) were administered to maintain elevated circulating corticosterone levels, as a previous study with a related species (*Rhinella icterica*) showed that transdermal application of corticosterone is sufficient to increase circulating corticosterone levels up to 1 h post application (Assis et al., 2017). Blood samples were obtained 2 h after the injections to measure circulating corticosterone levels, and the toads were euthanized with spleen tissue being removed and immediately frozen in liquid nitrogen for RNA-seq. Blood and tissue samples were stored at −80 °C until analysis.

2.2. Circulating corticosterone levels

Plasma samples were extracted according to the methods used by Mendonça et al. (1996). The samples were resuspended in EIA buffer and corticosterone concentrations were assessed using EIA kit (CORT catalog number ADI-900-097 - Enzo® Life Sciences). Intra-assay variation was 9.81%. Sensitivity of the assay was 44.68 pg/mL, and results of the treatments on corticosterone levels were analyzed using analysis of variance with Tukey's multiple comparison test.

2.3. RNA-seq

Spleens from the males in each treatment were pooled together into 2 groups of 3 males each, while the three females from each treatment were pooled together into one group, giving 3 pooled samples for each treatment. This was done to ensure adequate amounts of RNA would be obtained for sequencing, as well as preventing potential bias caused by