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Does physiological response to disease incur cost to reproductive ecology in a sexually dichromatic amphibian species?

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ABSTRACT

It is well known that the disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has contributed to amphibian declines worldwide. The impact of Bd varies, with some species being more susceptible to infection than others. Recent evidence has shown that Bd can have sub-lethal effects, whereby increases in stress hormones have been associated with infection. Could this increased stress response, which is a physiological adaptation that provides an increased resilience against Bd infection, potentially be a trade-off with important life-history traits such as reproduction? We studied this question in adult male frogs of a non-declining species (*Litoria wilcoxii*). Frogs were sampled for (1) seasonal hormone (testosterone and corticosterone), color and disease profiles, (2) the relationship between disease infection status and hormone levels or dorsal color, (3) subclinical effects of Bd by investigating disease load and hormone level, and (4) reproductive and stress hormone relationships independent of disease. Testosterone levels and color score varied seasonally (throughout the spring/summer months) while corticosterone levels remained stable. Frogs with high Bd prevalence had significantly higher corticosterone levels and lower testosterone levels compared to uninfected frogs, and no differences in color were observed. There was a significant positive correlation between disease load and corticosterone levels, and a significant negative relationship between disease load and testosterone. Our field data provides novel evidence that increased physiological stress response associated with Bd infection in wild frogs, could suppress reproduction by down-regulating gonadal hormones in amphibians, however the impacts on reproductive output is yet to be established.

1. Introduction

Animals are faced with the complex balance between investing energy into current reproduction and maintaining homeostasis (Greenberg and Wingfield, 1987; Hoffmann and Sgrò, 2011). Increased stress can upset this balance and lead to detrimental effects on the immune system and reproductive output (Guillette et al., 1995; Gustafsson et al., 1994). The fundamental question remaining is whether the physiological adjustments (such as increased stress responses) made by animals for coping against extreme environmental changes or disease, could incur major costs to reproduction or other vital fitness traits. Conservation physiology tools, such as non-invasive steroid monitoring, together with molecular disease surveillance and ecological methods will be important to understanding the fitness consequences of increased stress and disease in animals.

Chytridiomycosis is a disease caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (Bd) that has led to declines and extinctions of native amphibian populations across the globe (Berger et al., 1998). In spite of these mass declines, some amphibian species are seemingly able to persist, despite being highly susceptible to infection (Retallick et al., 2004). This leads to the question of the sub-lethal effects of Bd and what impacts it may have on a species, in particular what effects it may have on reproduction. It has been recently established that stress hormone levels increase (physiological stress response) in Bd infected amphibians (adults and tadpoles), with similar responses observed in highly susceptible non-declining species and susceptible declining species (Gabor et al., 2013; Gabor et al., 2015; Kindermann et al., 2012; Peterson et al., 2013).

The unanswered key questions within are 1) whether increased physiological stress responses under disease condition are a physiological mechanism to cope against infections in wild frogs, and 2) if increased Bd and stress levels are linked to diminished reproductive output in the species (Belden and Kiesecker, 2005; Reeve et al., 2013; Searle et al., 2014). Therefore, field based experimental studies on the interactions between disease, reproductive and stress hormones,

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and reproductive ecology of amphibians are needed to answer both questions.

The main stress hormone in amphibians, corticosterone (CORT) regulates homeostasis by re-directing energy away from non-essential functions and therefore assists survival of the organism (Romero, 2002); however prolonged elevated levels can have adverse effects on animal fitness (Moore and Jessop, 2003). CORT can either 1) suppress reproduction by downregulating gonadal hormones such as testosterone, 2) suppress reproductive behavior by acting directly on the central nervous system (Biswas et al., 2000; Gore et al., 2006; Moore and Miller, 1984; Moore and Zoeller, 1985) or 3) have a positive influence on reproduction by activating energy stores (Moore and Jessop, 2003). In turn reproductive hormones including testosterone can have secondary effects on animal immunity by negatively influencing immune function (Evans et al., 2000; Saad et al., 1990). This relationship is often due to a trade-off between investment into secondary sexual traits or mounting an immune response [immunocompetence handicap hypothesis] (Folstad and Karter, 1992). In the case of severe disease outbreaks animals may increase reproductive output to make terminal investment into reproduction (Chatfield et al., 2013). However several studies in amphibians have reported no significant relationships between reproductive hormones such as testosterone and disease (Dare and Forbes, 2009; Forbes et al., 2004; Stice, 2009).

With these differences existing in the literature, gaining a more comprehensive understanding of this interaction within one system (in this case the relationship between Bd, CORT, testosterone, and secondary sexual signals) can give greater insight into the complexities in others.

Litoria wilcoxii (Anura: Hylidae) is a common, nocturnal species found along streams on the east coast of Queensland, Australia. The species is sexually dichromatic (males rapidly turn yellow during amplexus and females are completely brown in coloration) and dimorphic (females are much larger than males); during the breeding season (spring and summer) males will form large aggregations along stream edges where they call for prospective females (Anstis, 2013). The brilliant yellow color in male *L. wilcoxii* appears primarily during the breeding season and rapid changes occur during amplexus and are driven by adrenalin (Kindermann et al., 2014), however color changes from brown to yellow occur from juvenile to adult life stages [when males reach sexual maturity] (Kindermann pers. comm.) suggesting that this a secondary sexual signal and is likely driven by reproductive hormones such as testosterone (Ohta et al., 2008). *Litoria wilcoxii* are said to be a reservoir population as despite being infected with Bd their populations don't seem to be declining (Kindermann et al., 2013; Kriger et al., 2007; Retallick et al., 2004).

Herein, we investigated the impact of Bd on hormone balance and seasonal reproductive coloration to elucidate the sub-lethal impact of this pathogen on a non-declining amphibian. We achieve this by examining (1) seasonal hormone, color and disease profiles, (2) the relationship between disease infection status and hormone levels or color score, (3) subclinical effects of Bd by investigating disease load and hormone level and (4) reproductive and stress relationships independent of disease. Determining physiological mechanisms that regulate breeding (stress and reproductive hormones) and how they are influenced by the intensity of Bd infection will advance the understanding of how species are being affected by disease. It is important to investigate the associations between disease status and host stress physiology in amphibian species that are currently not showing declines as it provide insights into the sub-clinical effects of disease on reservoir host species.

2. Material and methods

2.1. Field collection

A population of *L. wilcoxii* located along a creek section in Numinbah Valley, South East Queensland (28.219°S, 153.232°E, 196 m altitude) was sampled at monthly intervals ($n = 30$ per month, except March $n = 20$, May $n = 13$ and June $= 9$), from September 2013 until August 2014. No samples were collected during July as frogs could not be located due to cooler temperatures (monthly average minimum 10.7 °C). It should be noted that these samples are different from those previously published in Kindermann et al. (2012). Field sampling started at dusk (approx. 1830 h) and continued until all visible frogs had been sampled (approx. 2330 h). We captured male frogs by hand using 25 × 25 cm freezer bags and urine samples were immediately (within 1 min) taken on capture. Urine was collected using an Eppendorf pippete inserted into the frog's cloaca (1–2 mm) to collect urine via capillary action, after which frogs were placed into a 30 cm^3 box and photographed. A new freezer bag was used for each frog to prevent cross contamination. Following this all frogs were swabbed for Bd as per established methods (Kriger and Hero, 2007). Briefly, a fine tipped cotton swab (MW100–100; Medical Wire & Equipment, Wiltshire, England) was swiped 5 times over: the ventral surface, each side of the ventral surface, each thigh (underside) and each foot (Bd swabbing took on average 1 min per frog). All frogs were toe-clipped (assigned unique toe-clip codes for identification), using the numeric system described in Hero (1989) so individuals could be tracked over time. Amphibians have been shown to physiologically recover from a short-term stress response to toe-clipping at around 72 h (Narayan et al., 2011b). No negative side effects were observed following toe-clipping and clipped toes on individuals were fully healed when recaptured (*pers. obs.*). Samples (frog urine and Bd swabs) were kept on ice packs in the field for a period of 5 h and transported back to the laboratory where they were stored in a −80 °C freezer until hormone assays and PCR molecular analysis of Bd swabs were completed.

2.2. Color analysis

Color analysis follows the methodology developed by Kindermann et al. (2013). We used a digital camera (Canon Powershot S5, Japan) to acquire RAW format photographs of each frog captured. Frogs were photographed immediately following urine collection. To make sure all photos were consistent the setting and distance from a frog were identical for each photograph (sync macro and flash at full, 30 cm from a frog). We also placed a Munsell 24 Color Checker Chart next to each frog so that photographs could be calibrated if there were differences in exposure (de Velasco and Tattersall, 2008; Tattersall et al., 2006). This non-invasive technique of color measurement has been successfully used previously (de Velasco and Tattersall, 2008; Kindermann et al., 2014; Kindermann et al., 2013; Tattersall et al., 2006). We used Adobe Photoshop C35 Extended (Adobe Systems, 2010) to correct and analyze images. The corrected photo was cropped using the crop tool so that the dorsal body surface (rectangle from head to back legs) of the frog can be analyzed. Any pure white pixels (that were a result of flash reflection) were removed using the magic wand tool. The average RGB value for the cut-out section was calculated in Photoshop (Ohta et al., 2008). We then applied principal components analysis (PCA) using the statistical programing environment R (Rdevelopmentcoreteam, 2011, version 3.1.3) to reduce the three dimensions (RGB) to one dimension that explained a color range from brown [−3] to yellow [+3] (Kindermann et al., 2014; Kindermann et al., 2013; Vásquez and Pfennig, 2007; Wang and Shaffer, 2008).

2.3. Laboratory work

Each urine sample was analyzed using an enzyme immunoassay (EIA) for CORT and testosterone. CORT and testosterone metabolite concentrations were quantified using EIA methods previously described (Kindermann et al., 2012; Narayan et al., 2011b). Urinary CORT metabolite concentrations in *L. wilcoxii* urine were determined using a polyclonal anti-CORT antiserum (CJM06, UC Davis California) diluted 1:45,000 horseradish peroxidase-conjugated CORT label diluted 1:120,000 and CORT standards (1.56–400 pg $well^{-1}$). Urinary testosterone metabolite