Physiological effects of chytridiomycosis, a cause of amphibian population declines

by

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Abstract

Emerging infectious diseases (EIDs) of wildlife can have devastating impacts on biodiversity. The fungal disease, chytridiomycosis, caused by *Batrachochytrium dendrobatidis (Bd)* is implicated with amphibian population declines the world over. As is the case with many EIDs of wildlife, pathogenesis of chytridiomycosis is somewhat unclear. Pathogenesis involves disruption of cutaneous ion uptake, decreased plasma ions, and asystolic cardiac arrest, as well as seemingly unrelated effects on leukocytes, skin shedding, and appetite. In this dissertation I, along with the help of many collaborators, suggest that infection-induced decreases in plasma ions initiate a stress response which may mediate some of the deleterious effects observed during disease development. In chapters one and two, physiological parameters were monitored during an outbreak and a controlled infection, respectively, of *Bd* in a laboratory colony of *Litoria caerulea*. Taken together, it was observed that prior to becoming diseased, infected frogs experienced decreased plasma sodium and potassium, appetite, and body mass, as well as increased standard metabolic rate and skin shedding. When infected frogs became diseased, they contained even fewer plasma ions, as well as increased plasma corticosterone (CORT; a stress hormone) and altered white blood cell profiles. These individuals also had continued elevated standard metabolic rate and decreased body condition. In chapter three, it was determined that CORT increases standard metabolic rate in *L. caerulea*, representing the first time this effect has been observed in an anuran amphibian. Collectively, this dissertation suggests that stress physiology plays a role in chytridiomycosis.

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Table of Contents

List of Figures

List of Abbreviations

ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
Bd	*Batrachochytrium dendrobatidis*
CORT	Corticosterone
DPI	Days post infection
EID	Emerging infectious disease
NL ratio	Neutrophil-lymphocyte ratio
PCR	Polymerase chain reaction
PLSD	Protected least significant difference
RMR	Resting metabolic rate
SEM	Standard error of the mean
WBC	White blood cell

Introduction

Global biodiversity continues to decrease at an alarming rate (Butchart et al., 2010). Exploitation, global climate change, habitat loss, invasive species, anthropogenic contaminants, and emerging infectious diseases, as well as interactions among these factors have been linked to biodiversity loss (Smith et al., 2006). In vertebrates, the evolutionarily conserved stress response potentially mediates these effects, because it is one of the mechanisms by which vertebrates modulate responses to their environment (Wingfield et al., 1998). Besides regulating stress responses, the hypothalamic-pituitary-adrenal (-interrenal in amphibians) axis (stress axis), also regulates essential physiological functions (e.g. blood pressure, ion balance, blood glucose, immunity, metabolism, and reproduction; reviewed in (Sapolsky et al., 2000). Many of the above effects are mediated by glucocorticoids, such as cortisol and, importantly to this dissertation, corticosterone (CORT). Acute stress responses are considered adaptive, causing short lived alterations to these physiological functions in favor of survival, but chronic stress responses can be maladaptive, leading to suppression of reproduction, increased metabolic rate, and altered immunity (which can potentially increase susceptibility to disease; (Elenkov and Chrousos, 1999; Sapolsky et al., 2000). These effects can cause mortality or reduced fitness, and, thus, contribute to population declines and biodiversity loss. There is considerable evidence that many of the factors that lead to biodiversity loss also alter glucococorticoid secretion in vertebrates (Busch and Hayward, 2009).

Amphibians appear to be declining faster than other vertebrate groups (Stuart et al., 2004), and stress physiology may play an important role in these declines (Carey and Bryant,

1995; Pounds et al., 2006). Many of the factors linked to amphibian declines also alter CORT levels. For example, anthropogenic contaminants (Gendron et al., 1997; Glennemeier and Denver, 2001; Goulet and Hontela, 2003; Hayes et al., 2006; Hopkins et al., 1997; Hopkins et al., 1999; Larson et al., 1998; Peterson et al., 2009; Ward and Mendonça, 2006), infectious diseases (Belden and Kiesecker, 2005; Warne et al., 2011), habitat alteration (Denver, 1998; Newcomb Homan et al., 2003), and predation (Denver, 2009; Fraker et al., 2009) have been documented to alter CORT levels in amphibians. Like other vertebrates, glucocorticoids regulate many essential physiological processes in amphibians, such as plasma ion homeostasis (Brewer et al., 1980; De Ruyter and Stiffler, 1986; Heney and Stiffler, 1983; Middler et al., 1969; Stiffler et al., 1986; Yorio and Bentley, 1978), immunity (Belden and Kiesecker, 2005; Bennett et al., 1972; Bennett and Harbottle, 1968; Davis and Maerz, 2010; Garrido et al., 1987) metabolism (Wack et al., 2012), appetite (Crespi and Denver, 2005; Crespi et al., 2004) and skin shedding (Budtz, 1979; Jørgensen and Larsen, 1961, 1964; Stefano and Donoso, 1964). CORT also contributes to essential life history events, such as metamorphosis (Denver, 2009) and reproduction (Moore and Jessop, 2003). Since many of the factors that contribute to population declines are pervasive within amphibian habitats, populations may become chronically stressed and experience maladaptive effects on immunity, metabolism, and reproduction that contribute to long term population declines.

Chytridiomycosis, a disease caused by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), has caused amphibian declines the world over (Berger et al., 1998; Lips et al., 2006; Skerratt et al., 2007; Vredenburg et al., 2010) and, like other amphibian decline factors, potentially influences stress physiology. Although *Bd* is not known to alter CORT levels, it disrupts several physiological processes known to be influenced by glucocorticoids in

amphibians. Infection with *Bd* alters plasma ion homeostasis (Voyles et al., 2007; Voyles et al., 2009), immunity (Davis et al., 2010; Woodhams et al., 2007), appetite (Nichols et al., 2001; Voyles et al., 2009), and skin shedding (Nichols et al., 2001; Voyles et al., 2009).

Within this dissertation, we suggest that several of the detrimental effects of *Bd* infection may be mediated by CORT. *Bd* infects the superficial epidermis of post-metamorphic amphibians (Berger et al., 1998; Longcore et al., 1999), an organ that regulates ion homeostasis (Feder and Burggren, 1992). When amphibians become diseased (i.e. display clinical signs of disease), sodium uptake across the skin is disrupted and likely causes hyponatremia (reduced plasma sodium; Voyles et al., 2007; Voyles et al., 2009). *Bd*-induced hyponatremia likely increases CORT levels, because hyponatremia increases CORT levels in non-diseased amphibians (Stiffler et al., 1986). Increased CORT levels may function toward rebalancing plasma sodium in *Bd*-infected amphibians, because treatment with CORT can correct hyponatremia in non-diseased amphibians (Heney and Stiffler, 1983). However, if plasma sodium levels do not return to homeostatic levels, CORT levels may become chronically elevated in an attempt to regulate sodium levels. We predicted that increased CORT secretion during infection with *Bd* should correspond with previously observed effects on plasma ions, immunity, appetite, and skin shedding. We also predicted, given the known effects of CORT in amphibians, increases in CORT should also correspond with changes in body condition, body mass, and standard metabolic rate.

In chapter 1, we observed the physiological effects of a laboratory outbreak of *Bd* on Australian Green Tree Frogs, *Litoria caerulea*. In chapter 2, we observed the physiological effects of a controlled laboratory infection of *Bd* on *L. caerulea* at various time points throughout infection. Finally, in chapter 3, we verify that CORT increases metabolic rate in this species.

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Chapter 1. The pathogenesis of the deadly amphibian disease, chytridiomycosis, suggests development of a stress response.

Abstract

Chytridiomycosis, a disease caused by *Batrachochytrium dendrobatidis (Bd)*, has contributed to worldwide amphibian population declines; however, the pathogenesis of this disease is still somewhat unclear. Previous studies suggest that infection disrupts cutaneous sodium channels, which leads to hyponatremia and cardiac failure. However, infection is also correlated with unexplained effects on appetite, skin shedding, and white blood cell (WBC) profiles. Glucocorticoid hormones may be the biochemical connection between these disparate effects, because they regulate ion homeostasis and can also influence appetite, skin shedding, and WBCs. During a laboratory outbreak of *Bd* in Australian Green Tree Frogs, *Litoria caerulea*, we compared frogs showing clinical signs of chytridiomycosis to infected frogs showing no signs of disease and determined that diseased frogs contained elevated baseline corticosterone (CORT), decreased plasma sodium and potassium, and WBC profiles that paralleled those observed following CORT treatment in other studies. Diseased frogs also showed evidence of poorer body condition and elevated metabolic rates compared with frogs showing no signs of disease, as predicted by the metabolic effects of CORT on metabolic rate. Prior to displaying signs of disease, we also observed changes in appetite, body mass, and the presence of shed skin associated with infected but not yet diseased frogs. Collectively, these results suggest that

elevated baseline CORT is associated with *Bd* infections and may mediate some of the deleterious effects observed during disease development.

1. Introduction

Emerging infectious diseases (EIDs) of wildlife can have profound effects on animal biodiversity (Harvell et al., 1999; Lips et al., 2006); however, little is known about the pathogenesis of wildlife EIDs (Daszak et al., 2001). Since wildlife EIDs are often associated with anthropogenic and environmental stressors, pathogenesis is likely influenced by the host's response to stressors (Acevedo-Whitehouse and Duffus, 2009; Daszak et al., 2001; Dobson and Foufopoulos, 2001; Rachowicz et al., 2005). The evolutionarily conserved stress response is one of the mechanisms by which vertebrates modulate responses to these stressors (Wingfield et al., 1998). The stress response is of interest in a disease context, because it is mediated by glucocorticoid hormones that are known to affect susceptibility to infection (Elenkov and Chrousos, 1999).

Glucocorticoids influence a suite of physiological functions in vertebrates, including reproduction, development, blood ion homeostasis, metabolism, appetite, growth, and, importantly in the context of disease, immunity (Sapolsky et al., 2000). While much is known about how glucocorticoids influence physiological function in non-diseased animals, much less is known about how glucocorticoids influence the same physiological functions in diseased animals. To our knowledge only one such study has been conducted in wild vertebrates. Warne et al. (2011) exposed *Rana sylvatica* to ranaviruses and observed an increase in corticosterone (CORT; the most abundant amphibian glucocorticoid stress hormone) concentration and accelerated developmental changes consistent with the effects of endogenous and exogenous elevations of CORT in non-diseased amphibians.

Chytridiomycosis, a disease caused by the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has contributed to worldwide amphibian population declines. It is considered to be a significant threat to global amphibian biodiversity (Berger et al., 1998; Kilpatrick et al., 2010; Lips et al., 2006; Skerratt et al., 2007). Chytridiomycosis, like CORT, influences blood ion homeostasis, appetite, skin shedding, and immunity. Specifically, *Bd* disrupts sodium channels in the host's epidermis, which leads to hyponatremia and cardiac failure (Voyles et al., 2009). *Bd* also suppresses appetite (Nichols et al., 2001; Voyles et al., 2009), disrupts normal skin shedding (Nichols et al., 2001; Voyles et al., 2009), and causes alterations in leukocyte abundances in adult and larval anurans (Davis et al., 2010; Woodhams et al., 2007). Yet there are no studies that have attempted to document what hormones may be mediating these changes in blood ions, behavior, shedding, and leukocyte abundances.

Glucocorticoids may mediate the aforementioned effects of *Bd* infection. In amphibians, glucocorticoids are critical regulators of blood ion homeostasis (Brewer et al., 1980; De Ruyter and Stiffler, 1986; Heney and Stiffler, 1983; Middler et al., 1969; Stiffler et al., 1986; Yorio and Bentley, 1978), appetite (Crespi and Denver, 2005; Crespi et al., 2004), skin shedding (Budtz, 1979; Jørgensen and Larsen, 1961, 1964; Stefano and Donoso, 1964), and leukocytes (Belden and Kiesecker, 2005; Bennett et al., 1972; Bennett and Harbottle, 1968; Davis and Maerz, 2010; Garrido et al., 1987). A normal, adaptive, regulatory mechanism to maintain sodium homeostasis is likely a moderate, transitory elevation in CORT secretion to increase cutaneous uptake of sodium as well as digestive uptake (facilitated by increased appetite; Crespi and Denver, 2005; Heney and Stiffler, 1983; Stiffler et al., 1986). Since *Bd* infection directly compromises cutaneous sodium channels, a greater and greater elevation of CORT could occur in an attempt to maintain ion homeostasis. However, high concentrations of glucocorticoids can

become maladaptive, altering immune responses (Dhabhar and McEwen, 1997; Munck et al., 1984), increasing metabolic rate (DuRant et al., 2008), as well as actually suppressing appetite (Bernier, 2006), the latter effect further exacerbating ion imbalance, leading to unsustainable blood sodium levels and cardiac failure. Thus, CORT, in its regulatory role of maintaining ion homeostasis, is secreted in response to *Bd* infection, and could contribute to *Bd*-induced mortality.

The goal of this chapter was to determine whether *Bd* infection influences CORT levels and whether CORT profiles are associated with novel, as well as previously described effects of *Bd* infection. We documented the relationship of *Bd* infection to plasma corticosterone, sodium, and potassium concentrations; food intake; skin shedding; and leukocyte profiles during an outbreak of *Bd* in a laboratory colony of recently wild Australian Green Tree Frogs (*Litoria caerulea*). Since both CORT and disease influence energy balance, we also monitored metabolic rate, body condition, and body mass.

2. Materials and Methods

2.1 Laboratory outbreak and experimental design

Fifty one *Litoria caerulea* were obtained commercially (Tri Reptile, Miami, FL), in autumn of 2009. Frogs were recently (i.e., within two weeks) collected from the wild in Indonesia. Over the next two months six frogs died. Forty seven additional frogs were obtained from the same source three months later. Within a month, the frogs from the second shipment became ill and died at a rapid rate (i.e., 13 died within 13 days). Shed skin from individuals showing clinical signs of chytridiomycosis (e.g., listlessness, odd body posture, and skin discoloration; (Berger et al., 2005) was viewed under a light microscope and *Bd* was present in all samples viewed.

At this point, disease status [i.e., individuals displaying clinical signs of chytridiomycosis (diseased) or individuals not displaying clinical signs (non-diseased)] was monitored daily and food intake was assessed in all remaining non-diseased individuals (n=79). When an individual became diseased, the diseased frog and two randomly selected non-diseased individuals were swabbed (to quantify *Bd* zoospores), pithed, and bled (within 3 min of handling). Frogs were sacrificed in a separate area of the animal care facility to minimize disruption of the rest of the frogs in the colony. Preliminary statistical analyses suggested that order of sacrifice, duration of bleed, and time of bleeding had no significant effects on CORT and white blood cells ($P<0.05$). Several drops of whole blood were used to make blood smears for enumeration of leukocytes. The remaining blood was centrifuged for 4 min at 3,500 x g and the plasma was frozen and stored at -20º C for later use in corticosterone radioimmunoassay.

2.2 Animal care

Amphibians were housed individually in plastic containers (17 cm x 17 cm x 17 cm) in which paper towels saturated with well water were used as substrate. Wet paper towels were replaced twice each week for the duration of the study. Animals were fed as stated below under "food intake". Light was provided by full spectrum light bulbs on a 12:12 light/dark cycle. Room temperature was maintained by a thermostat at ~22° C.

2.3 Bd zoospore burden

Frogs were swabbed in a standardized fashion by lightly brushing a sterile cotton swab (Medical Wire & Equipment, MW113) 10 times over the sides, venter, and ventral surface of the thighs and 5 times over the underside of each foot (Kriger et al., 2006). Zoospore equivalents were determined by standard extraction and quantitative PCR techniques (Boyle et al., 2004; Ramsey et al., 2010). Nucleic acids were extracted by adding 60 µl of PrepMan Ultra (Applied

Biosystems, Foster City, CA) and 30-35 mg of Zirconium/silica beads (0.5 mm, Biospec Products, Bartlesville, OK) to the tip of each swab. Samples were homogenized for 45 s in a Mini Beadbeater (MP Bio, Solon, OH) and centrifuged for 30 s at 15,000 x g. After a second homogenization and centrifugation, the samples were boiled for 10 min, returned to room temperature for 2 min, and centrifuged at 15,000 x g for 3 min. Nucleic acids in the supernatant were removed for real-time PCR. Samples were loaded into an Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA) for 40 cycles of 95º C for 10 min, 95º C for 30 s, 55º C for 1 min, and 72º C for 1 min. Zoospore equivalents were determined using a standard curve and indicate *Bd* burden.

2.4 Ion analyses

Plasma prepared by centrifugation of whole blood for 4 min at 3,500 x g to remove red cells, was analyzed by Inductively Coupled Plasma with Optical Emission Spectroscopy (ICP-OES, Perkin Elmer 7100 DV, Waltham, MA) with simultaneous measurement of Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Na, P, S, Zn. Equal volume of plasma was diluted into ultra-pure, metal-free water (MilliQ, Millipore) then centrifuged at 13,000 x g to remove particulates and then introduced directly into the instrument argon plasma using a cyclonic nebulizer. Metal concentrations are determined comparing emission intensities to a standard curve created from certified metal standards (SPEX, Metuchen, NJ). Standard curves were confirmed by re-analysis of standard solutions diluted in a matrix equivalent to the sample. Individual readings are the average of two intensity measurements varied by less than 5%. Repeated analysis of individual samples showed less than 5 % variability.

2.5 Radioimmunoassay

Plasma corticosterone concentrations were determined by radioimmunoassay as described by Mendonça et al. (1996). All samples were run in one assay. Extraction efficiency was 81% and intraassay variation was 19.8%.

2.6 Food intake and body mass

Frogs were weighed weekly from the time they arrived in the laboratory. Once a week, for two weeks prior to sacrifice, each animal was weighed and fed approximately 10% of their body weight in 2.5 week old crickets coated in vitamin dust. All crickets not consumed were weighed after 24 hours. Food intake was determined as the mass of the crickets not consumed subtracted from the original mass of crickets given to the frog.

2.7 Shed skin collection

A subsample of frogs and bins were examined daily for the presence of shed skin. Shed skin was removed if it was observed on amphibians or within their containers. Dates in which skin was found on a frog or within its container were recorded. When frogs were sacrificed, the number of days, within the previous seven days, the frogs had shed skin on their body or within their container was determined. This value is referred to hereafter as "skin presence frequency".

2.8 Relative leukocyte numbers

Dried blood smears were stained with a Hema 3 kit (Fisher scientific, Kalamazoo, MI) and viewed under a light microscope. Slides were read in a standard zig-zag fashion. One hundred leukocytes were observed and the number of neutrophils, lymphocytes, eosinophils, monocytes, and basophils were recorded. Leukocytes were identified following Turner (1988) and Hadji-Azimi et al. (1987).

2.9 Respirometry and body condition

Closed system respirometry was used to measure resting metabolic rate (oxygen consumption) 1 day prior to sacrifice following the methods of Ward et al. (2006) with the following changes. Prior to being placed in individual respirometry chambers (140 ml syringes, Monoject, Sherwood Medical Industries, Ballymoney, UK), the venter of each frog was blotted dry with a paper towel and the bladder of each frog was voided by gently depressing the abdomen. Frogs were acclimated in their respirometry chamber for at least 45 min in a darkened incubator (22° C). Frogs were incubated for ~50 min in a darkened incubator (22° C). Any frogs that urinated or defecated during incubation were excluded from analyses. Frogs were weighed and their total body length was determined following respirometry.

2.10 Statistical analyses

Bd burden, plasma corticosterone, sodium, and potassium, food intake, skin presence frequency were compared relative to disease status with analyses of variance (ANOVAs). Oxygen consumption was compared between disease states with an analysis of covariance (ANCOVA) with disease state as the independent variable, oxygen consumed as the dependent variable, and body mass as the covariate. Since body mass influences oxygen consumption, oxygen consumption is presented as least squared means, corrected for body mass. Body condition was estimated as the residuals obtained by regressing body mass against total body length. Body condition was compared between disease states with an ANCOVA, with disease status as the independent variable, body mass as the dependent variable, and total body length as the covariate. Change in body mass was compared relative to disease status with a repeated measures ANOVA. Relative leukocyte numbers were compared relative to disease status with a multivariate analysis of variance (MANOVA). Sheffe's range tests were conducted for all a posteriori comparisons. We were unable to monitor changes over time for several variables;

however, when diseased and non-diseased frogs were sacrificed *Bd* burden was highly variable across all frogs (ranging from 0 to millions of zoospores per frog), suggesting that each frog was at a different point within disease progression. We used segmented regression to determine the threshold *Bd* burden at which the trend of corticosterone, lymphocytes, and eosinophils changed significantly, regardless of disease status (Seber and Wild, 1989). SAS (SAS institute, version 9.2) was used for the oxygen consumption ANCOVA (PROC GLM) and all segmented regressions (PROC NLIN). StatView for Windows (SAS institute, version 5.0.1) was used for all other statistical analyses.

3. Results

3.1 Measures of pre-diseased frogs

Frogs that eventually became diseased lost significantly more weight than frogs that remained non-diseased in the weeks prior to sacrifice (Repeated measures ANOVA, Disease status: $P<0.001$, $F_{1,21}=38.06$, Time: $P<0.001$, $F_{1,42}=12.25$, Disease status x Time: $P=0.2$; Figure 1.1). Individuals that eventually became diseased also consumed significantly less food one week prior to displaying clinical signs of disease compared to individuals that displayed no signs of disease (ANOVA, 0.022, $F_{1,21}=6.16$; Figure 1.2). During the week prior to sacrifice, shed skin was found on significantly more days within bins of frogs that eventually became diseased than within the bins of non-diseased frogs (ANOVA, $P<0.001$, $F_{1,21}=38.11$; Figure 1.2).

3.2 Measures of diseased frogs

Approximately 24 hours prior to sacrifice, frogs that displayed clinical signs of chytridiomycosis had significantly lower body conditions (ANCOVA, Disease status: $P<0.001$, $F_{1,20}=19.02$; Total body length: $P<0.001$, $F_{1,20}=273.43$). There was no disease status by total body length interaction ($P=0.22$). For ease of interpretation, these data are visually presented as

average residuals from a regression of body mass by total body length (Figure 1.3). Diseased individuals also consumed significantly more oxygen compared to non-diseased frogs (ANCOVA, Disease status: $P<0.001$, $F_{1,20}=24.52$, Body mass: $P=0.010$, $F_{1,20}=7.99$; Figure 1.3). There was no disease status by body mass interaction ($P=0.3$). On average, diseased frogs consumed more than twice the amount of oxygen non-diseased frogs consumed.

When sacrificed, swabs taken from diseased frogs contained significantly more *Bd* zoospore equivalents than swabs taken from non-diseased individuals (ANOVA, $P<0.001$, $F_{1,35}=19.66$; Figure 1.3). Although non-diseased individuals all had detectable levels of *Bd*, they contained approximately 1,000 times fewer zoospore equivalents than diseased individuals, on average.

Blood parameters taken at sacrifice also differed with disease status. Diseased frogs contained significantly fewer plasma electrolytes (ANOVA, Sodium: $P=0.049$, $F_{1,28}=4.24$, Potassium: $P=0.049$, $F_{1,28}=4.24$; Figure 1.3) and significantly greater concentrations of plasma corticosterone (ANOVA, $P=0.001$, $F_{1,34}=18.73$; Figure 1.3) compared with non-diseased frogs. Additionally, leukocyte abundances differed significantly between diseased and non-diseased individuals (MANOVA, $P<0.001$, $F_{1,20}=12.26$; Figure 1.4). Blood smears from diseased frogs contained significantly fewer lymphocytes and eosinophils and significantly more neutrophils than smears from non-diseased frogs (Sheffe's range tests, $P\leq0.002$).

3.3 Changes in corticosterone and leukocyte abundances at different Bd burdens

Since all individuals in the study contained different *Bd* burdens and were, thus, at different points in infection we used segmented regression to determine the zoospore intensity at which corticosterone, RMR, and lymphocyte abundances changed significantly (the zoospore

breakpoints). The zoospore breakpoints for corticosterone, RMR, and lymphocytes were 4,940; 4,066; and 10,778 zoospores, respectively (Figure 1.5).

4. Discussion

Individuals that displayed clinical signs of chytridiomycosis had significantly elevated baseline CORT, decreased plasma sodium and potassium, altered leukocyte profiles, increased metabolic rate, and decreased body condition compared with non-diseased individuals. The effects of *Bd* on leukocyte profiles and metabolic rate parallel those observed following CORT treatment in other studies (e.g. increased neutrophils and oxygen consumption and decreased lymphocytes and eosionphils; Belden and Kiesecker, 2005; Bennett et al., 1972; Bennett and Harbottle, 1968; Davis and Maerz, 2010; DuRant et al., 2008; Garrido et al., 1987; Wack et al., 2012). It is important to note that non-diseased individuals were also infected, but contained thousands of *Bd* zoospores while diseased individuals contained millions of zoospores, on average. When we plotted this broad range of *Bd* burdens (regardless of disease status) against CORT, RMR, and lymphocytes, segmented regressions indicated these three variables changed significantly at similar breakpoints (4,000-10,000 zoospores; Fig. 1.5).

Appetite suppression likely contributed to other effects we observed. For example, appetite suppression likely exacerbated hyponatremia because amphibians take up sodium via digestive as well as cutaneous routes (Feder and Burggren, 1992). Additionally, amphibians usually consume their shed skin, so appetite suppression also likely explains why we observed shed skin more often in the containers of frogs that eventually became diseased (Feder and Burggren, 1992). Finally, appetite suppression, coupled with an increased metabolic rate, may have contributed to the weight loss and poor body condition observed in frogs that eventually

became diseased. With no input of food, sick frogs must catabolize body tissues to meet their elevated respiratory demand, which results in weight loss and reduced body condition.

The levels of CORT in plasma were determined after development of several effects, so although infection was associated with decreased food intake, increased presence of shed skin, and weight loss, it is unclear whether increased CORT secretion was a cause or consequence of these parameters. We happened to be monitoring food intake, skin shedding, and weight loss as part of a separate experiment when the outbreak occurred, thus we did not monitor CORT throughout infection. Future studies are needed to determine when CORT increases during infection and whether CORT manipulation can alter chytridiomycosis pathogenesis.

It is unclear whether infection-induced glucocorticoid secretion is beneficial or maladaptive in vertebrates. Few studies have tested the effects of disease on baseline glucocorticoid levels in vertebrates (Al-Afaleq, 1998; Fast et al., 2006; Finstad et al., 2000; Fleming, 1997, 1998; Hanley and Stamps, 2002; Hermann et al., 1995; Laidley et al., 1988; Pickering and Christie, 1981; Raouf et al., 2006; Sures et al., 2001; Sures et al., 2002; Warne et al., 2011). Even fewer have observed how this may then lead to beneficial or deleterious physiological effects. To our knowledge, this is the first study that has assessed the effects of disease on baseline CORT levels in an adult amphibian (see Warne et al., 2010 for effects in tadpoles). Our data suggest that disease, at least chytridiomycosis, is likely a potent modulator of baseline CORT. Frogs displaying clinical signs of disease contained eight times more plasma CORT than non-diseased frogs. Average plasma CORT was 104 ng/ml in symptomatic frogs (maximum level of 270 ng/ml), rivaling the highest average levels of CORT observed in amphibians in response to stressors (Coddington and Cree, 1995; Gobbetti and Zerani, 1996; Hopkins et al., 1997; Jurani et al., 1973; Zerani et al., 1991). Given these findings and the large

number of emerging diseases in wildlife, we suggest that more studies focus on post-infection stress responses in wild animals.

Better understanding of the physiological effects of CORT, and its involvement in mediating factors that threaten the conservation status of amphibians (e.g. habitat destruction, global climate change, pollution, etc.) is needed. Specifically, there is a lack of data on how glucocorticoids influence metabolic rate and appetite in amphibians (Carr et al., 2002; Crespi et al., 2004). Our study provides data to suggest that CORT is associated with these factors, but controlled laboratory data are needed to complement this study. Understanding how amphibians respond to environmental change has become more urgent given recent amphibian population declines (Stuart et al., 2004). Several perturbations that potentially contribute to amphibian population declines have been linked to stress physiology [e.g. anthropogenic contaminants (Gendron et al., 1997; Glennemeier and Denver, 2001; Goulet and Hontela, 2003; Hayes et al., 2006; Hopkins et al., 1997; Hopkins et al., 1999; Larson et al., 1998; Peterson et al., 2009; Ward et al., 2007), disease (Warne et al., 2010), low habitat quality (Newcomb Homan et al., 2003), and habitat desiccation (Denver, 1998)]. Though the influence of anthropogenic contaminants on the stress axis has been relatively well studied in amphibians far less is known about how disease, habitat destruction, invasive predators, and climate change may influence stress physiology. Given the powerful and far reaching effects of glucocorticoids on wildlife life histories, understanding how these hormones mediate the interplay between environmental perturbations and life histories is essential to future conservation efforts.

Figures:

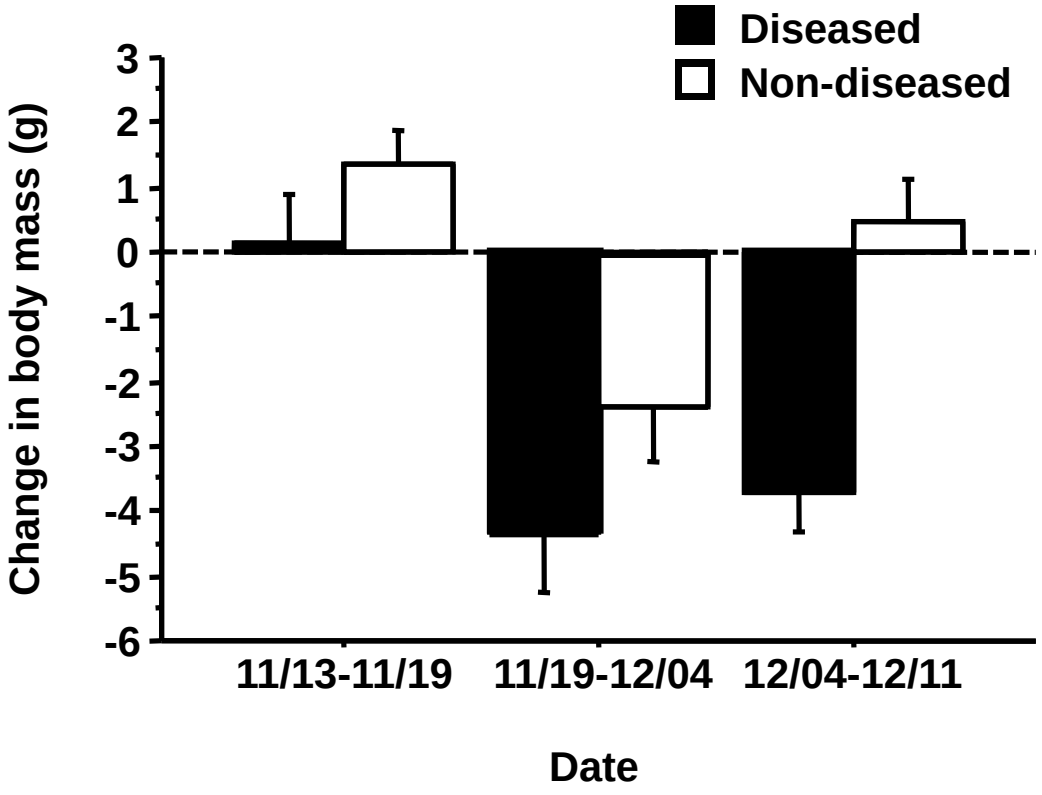

Fig. 1.1. Average change in body mass (±1 standard error) of *Litoria caerulea* that eventually became diseased (n=9) or remained non-diseased (n=14) for chytridiomycosis between dates leading up to sacrifice on 12/12/09. Disease statuses were statistically different (Repeated measures ANOVA, Disease status: $P<0.001$, $F_{1,21}=38.06$, Time: $P<0.001$, $F_{1,42}=12.25$, Disease status x Time: $P=0.2$).

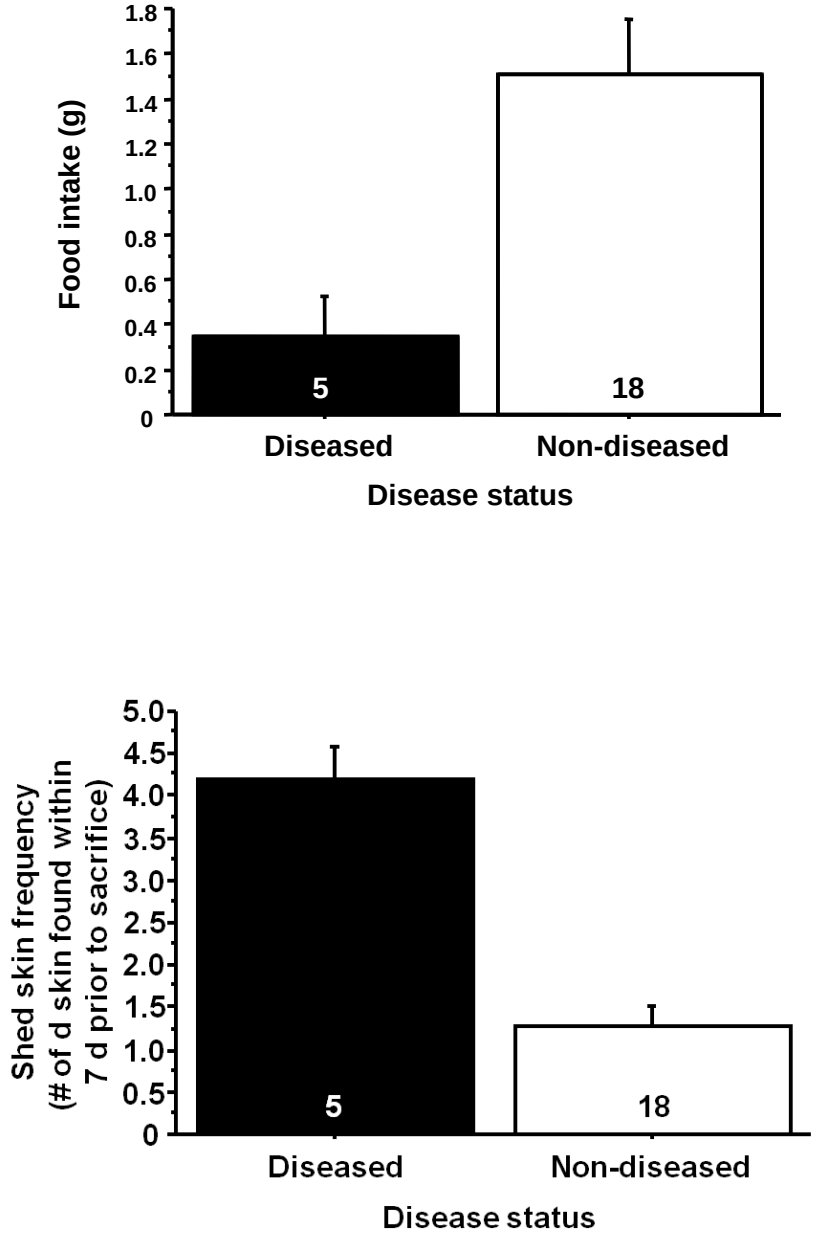

Fig. 1.2. Average food intake (top) and shed skin frequency (bottom) +1 standard error of *Litoria caerulea* that eventually became diseased or remained non-diseased at one week prior to sacrifice (top) and within the week leading up to sacrifice (bottom). Disease statuses were

statistically different (Food intake: ANOVA, P=0.022, $F_{1,21}$=6.16; shed skin frequency: ANOVA, P<0.001, $F_{1,21}$=38.11).

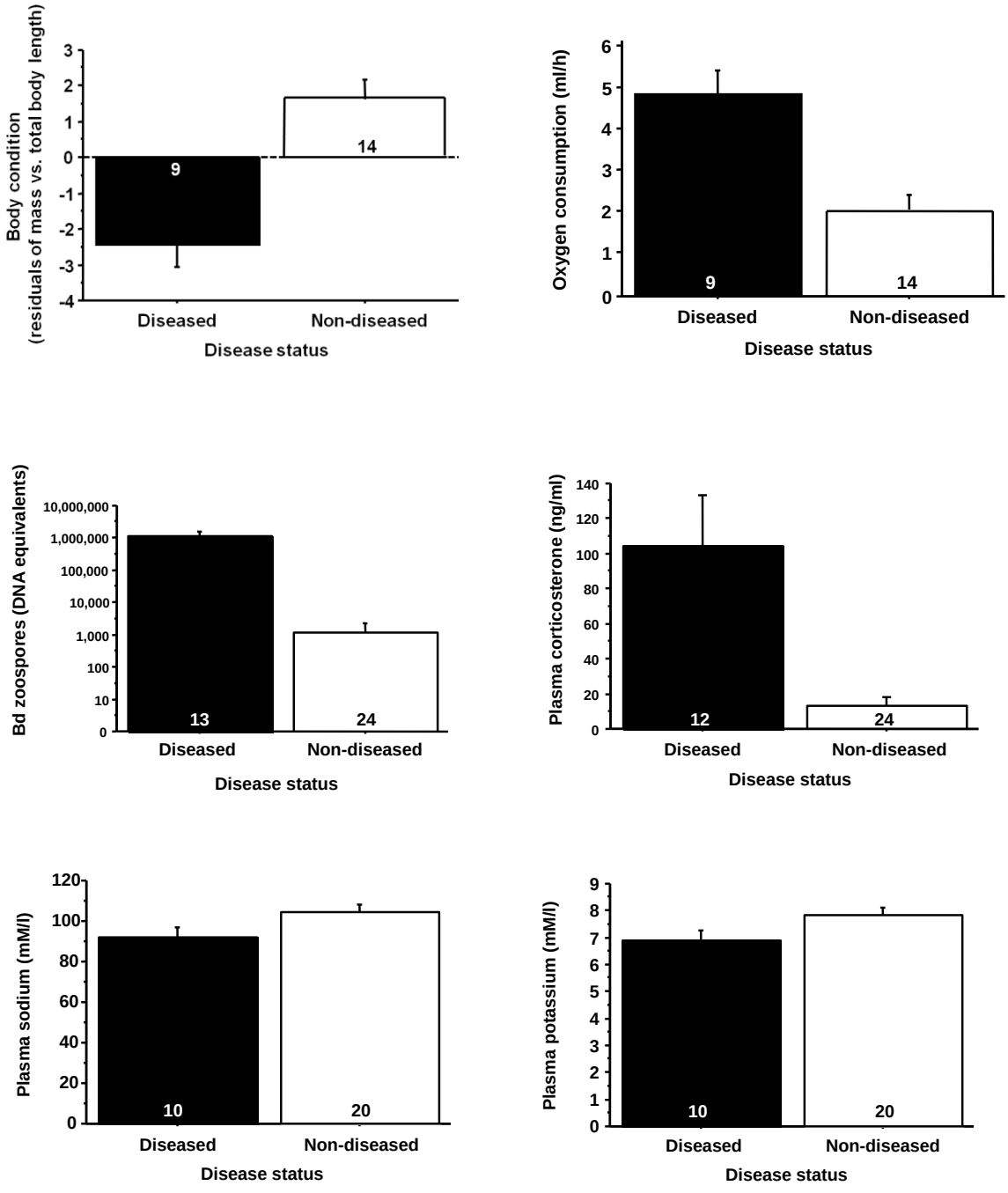

Fig. 1.3. Mean body condition, oxygen consumption, log+1 transformed *Bd* zoospores, plasma corticosterone, plasma sodium, and plasma potassium ± 1 standard error of *Litoria caerulea* that displayed clinical signs of disease (diseased) or did not display clinical signs of disease (non-

diseased). Disease statuses were significantly different for all measures (ANOVA/ANCOVA, $P<0.05$).

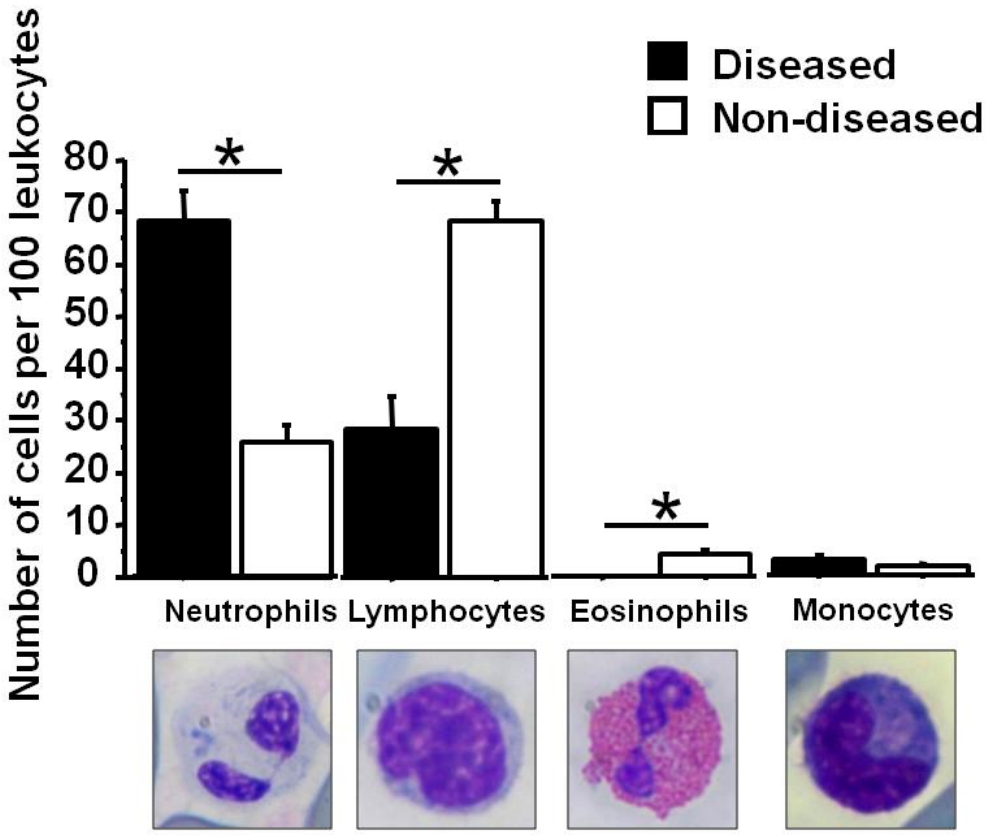

Fig. 1.4. Average relative abundances of neutrophils, lymphocytes, eosinophils, and monocytes per 100 leukocytes + 1 standard error from *Litoria caerulea* displaying clinical signs of chytridiomycosis (diseased, n=7) or not displaying clinical signs of disease (non-diseased, n=19). Leukocyte abundances were significantly different between groups (MANOVA, $P<0.001$, $F_{1,20}=12.26$). Asterisks denote significant differences. Blood smears from diseased frogs contained significantly fewer lymphocytes and eosinophils and significantly more neutrophils than smears from non-diseased frogs (Sheffe's range test, $P<0.001$). Average basophil abundances were part of the analysis but were excluded.

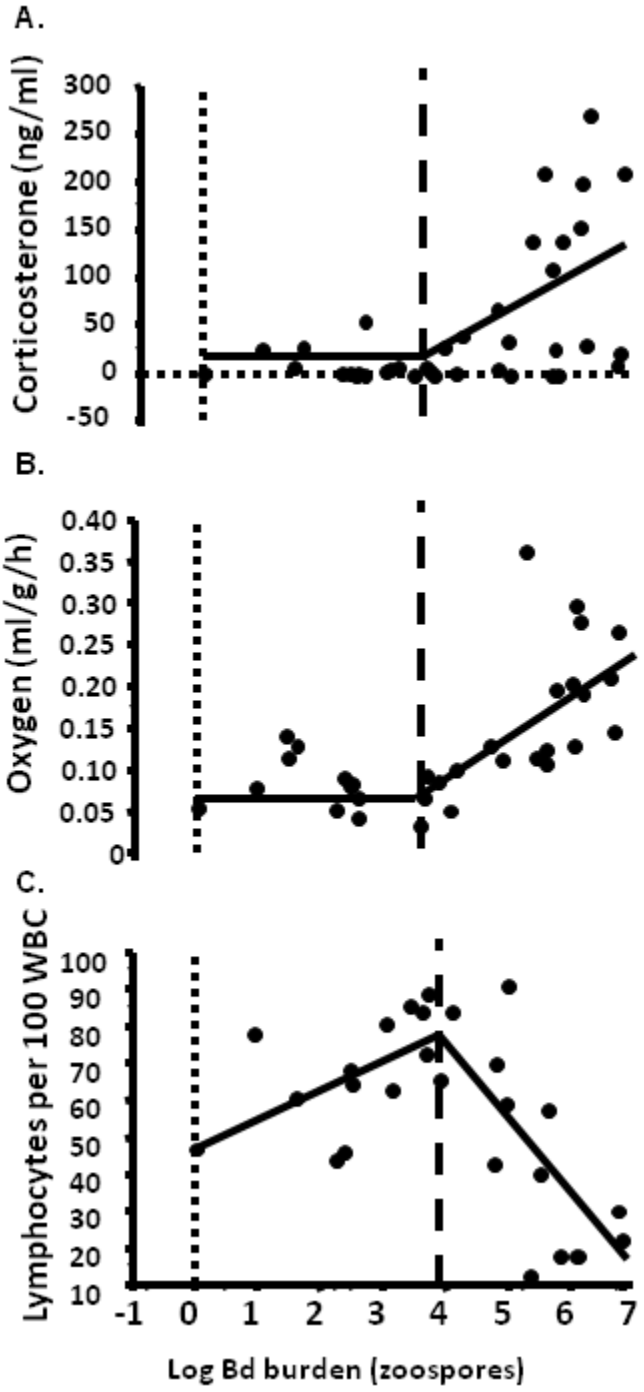

Fig. 1.5. Segmented regressions of (A) plasma corticosterone, (B) RMR, and (C) lymphocyte abundances of *Litoria caerulea* during an outbreak of chytridiomycosis. Horizontal and vertical

dotted lines indicate X and Y axes, respectively. Vertical dashed lines indicate breakpoints at which the dependent variables changed significantly. Black lines indicate the two segments fit to the data before and after the breakpoint. The zoospore breakpoints for corticosterone, RMR, and lymphocytes were 4,940; 4,066; and 10,778 zoospores, respectively. Data before and after the breakpoint were significantly different for all three variables (Segmented regression, $P<0.05$).

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Chapter 2. Physiological progression of the amphibian decline-causing disease, chytridiomycosis.

Abstract

The fungal disease, chytridiomycosis, is considered the largest infectious disease threat to global biodiversity because of its world-wide catastrophic effects on amphibian populations. Pathogenesis appears to involve complex interactions among physiological systems; however, relatively few studies have observed how the infectious agent, *Batrachochytrium dendrobatidis (Bd)*, alters host physiology. Even fewer studies have monitored physiological changes prior to and throughout infection. In order to evaluate physiological changes associated with *Bd* infection, we infected *Litoria caerulea* with *Bd* and measured changes in physiological variables prior to and throughout infection. Prior to showing clinical signs of disease, infected frogs first experienced hyponatremia and hypokalemia, followed by reduced food intake and body mass and increased metabolic rate. When infected frogs became diseased, they experienced further plasma ion depletion accompanied by increased baseline plasma glucocorticoid levels and neutrophil-lymphocyte ratios. We suggest that the combined effects of reduced cutaneous and digestive sodium uptake, greatly contribute to the fatal hyponatremia observed during chytridiomycosis.

1. Introduction

Chytridiomycosis, a disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), is linked to amphibian population declines world-wide. Pathogenesis of chytridiomycosis

involves disruption of cutaneous sodium channels, hyponatremia, and cardiac failure (Voyles et al., 2009). Additional described aspects of the fungal induced pathogenesis include increased baseline plasma corticosterone (CORT) levels (see Chapter 1 of this dissertation), altered numbers of circulating leukocytes (Davis et al., 2010; Woodhams et al., 2007a) increased metabolic rate (see chapter 1 of this dissertation), decreased appetite (Nichols et al., 2001; Voyles et al., 2009), alteration of normal skin shedding processes (see chapter 1 of this dissertation; Nichols et al., 2001; Voyles et al., 2009), weight loss (Harris et al., 2009; Retallick and Miera, 2007) and decreased body condition (see chapter 1 of this dissertation).

In amphibians, glucocorticoids (i.e., primarily CORT) routinely regulate a host of related physiological processes that are potentially associated with the previously observed aspects of chytridiomycosis pathogenesis, such as changes in plasma sodium homeostasis (Brewer et al., 1980; De Ruyter and Stiffler, 1986; Heney and Stiffler, 1983; Middler et al., 1969; Stiffler et al., 1986; Yorio and Bentley, 1978), immunity (Belden and Kiesecker, 2005; Bennett et al., 1972; Bennett and Harbottle, 1968; Davis and Maerz, 2010; Garrido et al., 1987) metabolism (Wack et al., 2012), appetite (Crespi and Denver, 2005; Crespi et al., 2004) and skin shedding (Budtz, 1979; Jørgensen and Larsen, 1961, 1964; Stefano and Donoso, 1964). Thus, CORT may be mediating aspects of pathogenesis. Non-pathogen-induced hyponatremia causes CORT secretion in amphibians in order to regulate plasma sodium concentration (Stiffler et al., 1986). Also, CORT treatment can correct non-pathogen-induced hyponatremia (Heney and Stiffler, 1983), so it is likely that pathogen-induced hyponatremia would also increase CORT levels in amphibians. If CORT is ineffective at rebalancing sodium homeostasis, then chronic activation of the stress axis may occur resulting in continued elevation in CORT leading to immunomodulation (Belden and Kiesecker, 2005; Bennett et al., 1972; Bennett and Harbottle, 1968; Davis and Maerz, 2010;

Garrido et al., 1987), elevated metabolic rate (Wack et al., 2012), and appetite suppression (Crespi et al., 2004). The latter effect would reduce digestive sodium uptake and further depress plasma sodium levels until they become too low to provide cardiac conductivity (Voyles et al., 2009). This set of events could, rather than help regulate sodium levels, further reduce sodium levels, thus exacerbating disease.

During a laboratory outbreak of *Bd* in the Australian Green Tree Frog, *Litoria caerulea*, we observed a significant elevation in baseline plasma CORT and contemporaneous changes in plasma sodium and potassium, leukocyte profiles, metabolism, food intake, and body mass (see chapter 1 of this dissertation). Most of these variables were only monitored at one time point within infection, making it difficult to determine when symptoms develop. Identification of when ion levels, CORT, and related variables change during infection would provide better documentation of when pathologies develop and when intervention could be beneficial. In an attempt to document when physiological changes occur we infected *L. caerulea* with *Bd*, and monitored changes in the aforementioned variables prior to and throughout infection. We predict that hyponatremia will be the first ill effect of infection, followed by alterations in plasma CORT, leukocyte profiles, metabolic rate, appetite, and weight loss.

2. Methods

2.1 Experimental design and blood collection

Litoria caerulea were obtained commercially (Tri-Reptile, Miami, FL) in summer 2010. Frogs were recently collected from the wild in Indonesia. All frogs were heat-treated following established protocols (Woodhams et al., 2003) to rid them of previous *Bd* infections. Although this treatment did not completely rid frogs of *Bd*, any retained infections remained light

throughout the study. Approximately two weeks after heat treatment, pre-infection measures were taken for a subset of frogs (n=8) for resting metabolic rate, body mass, and food intake. These same frogs were swabbed for *Bd* burden and sacrificed to obtain blood for plasma ions, plasma CORT, and blood leukocytes. On the same day, all remaining frogs were infected with methods detailed below. Resting metabolic rate, body mass, food intake, *Bd* burden, and blood parameters were assessed in subsets of frogs early in infection (3-9 days post infection; DPI), midway through infection (30-39 DPI), and late in infection (32-88 DPI). Early in infection and midway through infection amphibians were sampled for *Bd* burden and blood variables on specific days (i.e. 7 and 39 DPI, respectively), but late in infection these variables were assessed when individuals became clinically diseased (i.e. listless, odd body posture, and skin discoloration; (Berger et al., 2005)). When an individual became diseased it was sacrificed along with a control individual of similar body size. During sacrifice, the frog was swabbed (to quantify *Bd* zoospores), pithed, and bled (within 3 minutes of handling). Several drops of whole blood were used to make blood smears for enumeration of leukocytes. The remaining blood was centrifuged for 4 minutes at 3,500 x g and the plasma was frozen and stored at -20º C for later use in corticosterone radioimmunoassay and ion analyses.

2.2 Animal Care

Amphibians were housed individually in plastic containers (17 cm x 17 cm x 17 cm) containing paper towels saturated with well water. Wet paper towels were replaced twice a week for the duration of the study. Animals were fed as stated below under "food intake and body mass". Light was provided by full spectrum light bulbs on a 12:12 light/dark cycle. Room temperature was controlled by a thermostat (~22° C).

2.3 Bd culture and experimental infection

Batrachochytrium dendrobatidis cultures were maintained in 10% tryptone broth. Prior to infection 0.25 ml of broth was placed on agar plates. Following desiccation of the broth, plates were stored for a week at 16° C. Plates were then flooded with 3 ml of broth, which was poured off an hour later. *Bd* zoospores were counted using a hemacytometer and each frog was inoculated once with 20 µl of either broth containing approximately one million zoospores (*Bd*) or broth containing no zoospores (control).

2.4 Bd zoospore burden

Frogs were swabbed in a standardized fashion by lightly running a sterile cotton swab (Medical Wire & Equipment, MW113) 10 times over the sides, venter, and ventral thighs and 5 times over the underside of each foot (Kriger et al., 2006). Zoospore equivalents were determined by standard extraction and quantitative PCR techniques (Boyle et al., 2004; Ramsey et al., 2010). Nucleic acids were extracted by adding 60 µl of PrepMan Ultra (Applied Biosystems, Foster City, CA) and 30-35 mg of Zirconium/silica beads (0.5 mm, Biospec Products, Bartlesville, OK) to the tip of each swab. Samples were homogenized for 45 s in a Mini Beadbeater (MP Bio, Solon, OH) and centrifuged for 30 s at 15,000 x g. After a second homogenization and centrifugation, the samples were boiled for 10 min, returned to room temperature for 2 minutes, and centrifuged at 15,000 x g for 3 min. Nucleic acids in the supernatant were removed for real-time PCR. Samples were loaded into an Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA) for 40 cycles of 95º C for 10 min, 95º C for 30 s, 55º C for 1 min, and 72º C for 1 min. Zoospore equivalents were determined using a standard curve and indicate *Bd* burden.

2.5 Ion analyses

Plasma prepared by centrifugation of whole blood for 4 minutes at 3,500 x g to remove red blood cells, was analyzed by Inductively Coupled Plasma with Optical Emission Spectroscopy (ICP-OES, Perkin Elmer 7100 DV, Waltham, MA) with simultaneous measurement of Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Na, P, S, Zn. Equal volume of plasma was diluted into ultra-pure, metal-free water (MilliQ, Millipore) then centrifuged at 13,000 x g to remove particulates and then introduced directly into the instrument argon plasma using a cyclonic nebulizer. Metal concentrations are determined comparing emission intensities to a standard curve created from certified metal standards (SPEX, Metuchen, NJ). Standard curves were confirmed by re-analysis of standard solutions diluted in a matrix equivalent to the sample. Individual readings are the average of two intensity measurements varied by less than 5%. Repeated analysis of individual samples showed less the 5% variability.

2.6 Radioimmunoassay

Plasma corticosterone concentrations were determined by radioimmunoassay as described by Mendonça et al. (1996). All samples were run in three assays. Average extraction efficiency was 73.7%. Average intraassay variation was 10.9% and interassay variation was 13.3%.

2.7 Relative leukocyte counts

Dried blood smears were stained with a Hema 3 kit (Fisher scientific, Kalamazoo, MI) and viewed under a light microscope. Slides were read in a standard zig-zag fashion. One hundred leukocytes were observed and numbers of neutrophils, lymphocytes, eosinophils, monocytes, and basophils were recorded. Leukocytes were identified following Turner (1988) and Hadji-Azimi et al. (1987). Leukocyte data are presented as neutrophil-lymphocyte ratios (NL ratios).

2.8 Respirometry

Closed system respirometry was used to measure resting metabolic rate (oxygen consumption) 19 days prior to infection (pre-infection), early in infection (9 DPI), midway through infection (30 DPI), and late in infection (range: 30-51 days post infection and 5-8 days prior to sacrifice) following the methods of Ward et al. (2006) with the following changes. Frogs were acclimated in 140 ml syringes (Monoject, Sherwood Medical Industries, Ballymoney, UK) for at least 45 minutes in a darkened incubator (25°C). Frogs were incubated for ~50 minutes in a darkened incubator (25°C). Any frogs that urinated or defecated during incubation were excluded from analyses. Frogs were weighed following respirometry. Respirometry data are presented as ml of oxygen consumed per gram of body mass per hour.

2.9 Food intake and body mass

Body mass and food intake were determined on consecutive days 18 and 17 days prior to infection, early in infection (3 and 4 DPI), midway through infection (31 and 32 DPI), and late in infection (31-79 DPI), respectively. Body mass and food intake late in infection were assessed an average of 5 and 7 days prior to when frogs became diseased (range: 0-10 and 2-11), respectively. Each frog was weighed and fed approximately 10% of its body weight in 2.5 week old crickets coated in multi-vitamin dust. All crickets not consumed were weighed after 24 hours. Food intake was determined as the mass of the crickets not consumed subtracted from the original mass of crickets given to the frog.

2.10 Statistical analyses

Bd burden, plasma sodium, plasma corticosterone, neutrophil-lymphocyte ratios, and food intake were compared among time points for individuals sampled prior to infection and infected individuals with analyses of variance (ANOVAs). Oxygen consumption was compared

among time points with an analysis of covariance (ANCOVA) with time as the independent variable, oxygen consumed as the dependent variable, and body mass as the covariate. Since body mass influences oxygen consumption, oxygen consumption is presented as least squared means, corrected for body mass. Fisher's protected least significant difference (Fisher's PLSD) tests were used for all post-hoc comparisons among time points. An additional ANOVA was used to compare the above variables (except for oxygen consumption) between infected individuals that became diseased and controls. An additional ANCOVA was used to compare oxygen consumption between infected individuals that became diseased and controls (with body mass as the covariate). Corticosterone values were log+1 transformed prior to all statistical analyses to better fit the assumptions of ANOVA; however, non-transformed values are presented in the results and figures for ease of interpretation. Change in body mass and food intake prior to becoming diseased were compared between treatments with repeated measures ANOVAs. We used StatView for Windows (SAS institute, version 5.0.1) for all statistical analyses.

3. Results

3.1 Bd burden

Bd burden of infected frogs increased significantly throughout infection (ANOVA, P=0.001, $F_{3,27}$=7.15). Three of the eight frogs sampled prior to infection contained light *Bd* infections (range: 17-28 zoospore equivalents). Infected individuals at 7 and 39 days post infection contained moderate *Bd* burdens (mean: 13,870 and 18,700, respectively) that were significantly higher than individuals sampled prior to infection and significantly lower than diseased individuals, who contained an average of 16.5 million zoospore equivalents (range: 1.8-50.9

million; Fisher's PLSD post hoc tests $P \leq 0.001$). Control individuals sampled with diseased individuals contained significantly fewer zoospore equivalents than diseased individuals (ANOVA, P=0.029, $F_{1,12}=6.16$). Control individuals sampled at 7 and 39 days post sham-infection contained light *Bd* infections (average: 28 and 39, respectively).

3.2 Plasma ions

Plasma sodium and potassium of infected frogs decreased significantly throughout infection (ANOVA, $P<0.001$, $F_{3,19}=11.81$; ANOVA, P=0.001, $F_{3,19}=7.72$; respectively). Plasma of frogs bled at 39 days post infection contained 22% and 33% less sodium and potassium, respectively, than individuals sampled prior to infection, while plasma of diseased frogs contained 48% and 49% less sodium and potassium, respectively. Plasma of diseased frogs contained approximately half as much sodium as frogs bled prior to infection and one week post-infection (Fisher's PLSD, $P<0.05$). Infected frogs that became diseased also contained 47% less plasma sodium and potassium than control frogs sampled at the same time (ANOVA, $P<0.001$, $F_{1,9}=29.85$; ANOVA, $P<0.001$, $F_{1,9}=24.19$; respectively).

3.3 CORT

Baseline plasma CORT increased significantly throughout infection in infected individuals (ANOVA, P=0.015, $F_{3,24}=4.25$). When infected frogs became diseased they contained an average of 111 ng/ml CORT, representing a 7.6 fold increase compared to individuals sampled at 39 days post infection (Fisher's PLSD, $P \leq 0.019$) and a 25.7 fold increase compared to control individuals sampled concurrently (ANOVA, P=0.005, $F_{1,9}=13.68$).

3.4 NL ratios

Neutrophil-lymphocyte ratios increased throughout infection in infected frogs (ANOVA, P=0.047, $F_{3,20}=3.17$). Ratios remained low through 7 days post infection and were significantly

elevated in infected frogs that became diseased (Fisher's PLSD, $P<0.05$). Infected frogs that became diseased contained more than twice as many neutrophils compared to lymphocytes while control frogs at the same time point contained the opposite, more than twice as many lymphocytes compared to neutrophils (ANOVA, $P=0.005$, $F_{1,9}=13.69$).

3.5 Respirometry

Oxygen consumption increased significantly throughout infection in infected frogs (ANCOVA, Time: $P=0.023$, $F_{3,20}=3.97$; Body mass: $P=0.009$, $F_{1,20}=8.51$). There was no time by treatment interaction ($P=0.096$). Infected frogs sampled an average of 6 days prior to becoming diseased consumed significantly more oxygen than frogs evaluated prior to infection and nine days post infection (Fisher's PLSD, $P<0.05$). These same frogs also consumed 1.8 times more oxygen than control frogs sampled concurrently (ANCOVA, Treatment: $P=0.032$, $F_{1,5}=8.73$; Body mass: $P=0.4$, $F_{1,5}=0.76$). There was no treatment by body mass interaction ($P=0.9$).

3.6 Food intake

Food intake decreased in infected frogs over the course of the experiment (ANOVA, $P=0.009$, $F_{3,24}=4.90$). An average of seven days prior to displaying clinical signs of disease, infected individuals consumed significantly fewer grams of crickets than individuals sampled prior to infection and infected individuals sampled at four and 32 days post infection (Fisher's PLSD, $P<0.05$). Infected individuals sampled an average of seven days prior to displaying clinical signs of disease consumed approximately 7% the amount of crickets consumed by frogs sampled prior to infection (Fisher's PLSD, $p=0.002$) and control individuals sampled at the same time (ANOVA, $P<0.001$, $F_{1,9}=160.36$). Food intake was significantly lower for at least an average of 15 days prior to becoming diseased compared to control individuals samples concurrently

(Repeated measures ANOVA, Treatment: $P<0.0001$, $F_{1,7}=86.17$, Time: $P=0.3$, $F_{1,7}=1.06$, Treatment x Time: $p=0.4$, $F_{1,7}=0.91$).

3.7 Change in body mass

Infected frogs experienced significantly more change in mass throughout the experiment compared to control frogs (Repeated measures ANOVA, Treatment: $p=0.004$, $F_{1,12}=13.02$, Time: $p=0.024$, $F_{2,24}=4.38$, Treatment x Time: $p=0.8$, $F_{2,24}=0.22$). Control frogs gained mass up to 31 days post infection and then lost 0.5 grams between 31 days post infection and time points prior to sacrifice; whereas infected individuals lost 0.22 grams of mass between 3 and 31 days post infection and lost 2.9 grams between 31 days post infection and time points prior to sacrifice.

4. Discussion

This is the first study to document the timeline of endocrine, immune, and metabolic changes throughout *Bd* infection. In another experiment that monitored effects of *Bd* infection over time, Voyles et al. (2009) monitored changes in ions and major molecules found in the blood and urine. Both of these studies observed that *Litoria caerulea* exhibit little change in variables during the first 30-39 days post infection. Despite previous suggestion that inappetance may be the earliest clinical sign of disease (Nichols et al., 2001), we observed that hyponatremia and hypokalemia preceded changes in any other variables monitored in this study, including appetite. Unfortunately, it is unclear how many days this change in plasma ions predates changes in appetite. Our experimental design set certain sample times, but there was variation when animals displayed clinical signs of disease when they were sampled late in infection. Thus, we could not completely capture changes in plasma ions in the time before animals became diseased.

Bd burdens were not a good predictor of pathogenesis. *Bd* burdens of frogs already showing signs of hyponatremia and hypokalemia at 39 days post infection had highly variable *Bd* burdens (actual data: 50; 466; 2,714; 4,366; 15,156; and 89,448). For example, the individual with the lowest *Bd* burden (50 zoospore equivalents) contained the lowest amounts of plasma sodium and potassium (62 and 1.75 mM/l, respectively), yet previous data suggests that plasma sodium is positively correlated with *Bd* burden (Voyles et al., 2009). Even though *Bd* burdens may be informative for predicting epidemiology (Kinney et al., 2011; Vredenburg et al., 2010), our data suggest the use of *Bd* burdens in lab studies of pathogenesis would be less informative and not predictive.

Although hyponatremia and hypokalemia were the earliest physiological changes to occur, they took several weeks to manifest. We observed no changes in plasma ions at seven days post infection and Voyles et al. (2009) observed no changes at 30 days post infection, but in our study, by day 39, we observed significant decreases in both plasma sodium and potassium. Several factors may provide protection from *Bd*-induced ion loss. During these initial weeks of infection, epidermal antimicrobial peptides and anti-fungal bacteria, which have the ability to inhibit *Bd* growth *in vitro* (Harris et al., 2006; Woodhams et al., 2007a; Woodhams et al., 2007b), likely provide some protection to the skin. Once *Bd* colonizes the skin and individuals show signs of disease, sodium uptake across the skin is significantly suppressed, leading to plasma ion loss (Voyles et al., 2009). During hyponatremia and hypokalemia, corticoid hormones (i.e. CORT and aldosterone) function to rebalance plasma ion levels in amphibians (De Ruyter and Stiffler, 1986; Heney and Stiffler, 1983; Stiffler et al., 1986). In fact, at 39 days post infection, the individuals with the highest CORT levels (e.g., 27.4 and 46.9 ng/ml) contained plasma sodium levels (120 and 102 mM/l) similar to controls. These individuals also

displayed elevated NL ratios (1.6 and 3.8), and oxygen consumption (0.13 and 0.21 ml/g/hr) equivalent to those observed in frogs undergoing a stress response (Belden and Kiesecker, 2005; Bennett et al., 1972; Bennett and Harbottle, 1968; Coddington and Cree, 1995; Davis and Maerz, 2010; Garrido et al., 1987; Jurani et al., 1973). Although average CORT, NL ratios, and oxygen consumption were not significantly altered around 39 days post infection, these data points suggest that elevated CORT may be helping to rebalance plasma sodium, shifting NL ratios and increasing oxygen consumption. Since glucocorticoid treatment can correct hyponatremia in humans and amphibians (Davis et al., 1969; Heney and Stiffler, 1983; Kamoi et al., 1993; Merriam and Baer, 1980), future studies could test if CORT or aldosterone treatment potentially correct *Bd*-induced ion imbalances early in infection.

Our results suggest that the observed initial effects on plasma ions at 39 days post infection do not appear to be linked to food intake. At 39 days post infection, infected individuals contained 22% and 33% less plasma sodium and potassium, respectively, than individuals sampled prior to infection, even though, seven days earlier, they had consumed a "normal" amount of food (i.e. an amount of food similar to individuals sampled prior to infection and four days post infection). Although infected frogs apparently had a diet source of sodium, their plasma sodium levels decreased substantially, indicating that food intake could not completely compensate for decreased sodium input via the skin.

Although CORT secretion during *Bd* infection may initially rebalance sodium homeostasis to a certain extent, it is clear that these elevated levels are seemingly ineffective by the time individuals display clinical signs of disease. By the time individuals became diseased, CORT levels were 7.6 times higher than individuals sampled at 39 days post infection; however, plasma sodium levels continued to decrease. In fish and mammals, low glucocorticoids can

stimulate appetite, but stressors and high glucocorticoids can suppress appetite (Bernier, 2006; Dallman et al., 1993). Thus, the observed, potentially chronic elevation of CORT or other aspects of the stress response, such as corticotropin-releasing factor (Crespi et al., 2004), would suppress appetite and, thus, decrease digestive sodium uptake, worsening the animal's sodium balance instead of rescuing it. Although we observed that food intake in infected frogs was significantly suppressed at least 15 days prior to when they became diseased, we were unable to determine whether CORT caused this suppression, due to limitations of our data. In order to determine if CORT suppresses appetite during infection with *Bd*, future studies documenting food intake and CORT levels late in infection are needed.

Our data and data from the first chapter of this dissertation suggest that by the time frogs display clinical signs of disease they are secreting glucocorticoids that are at levels that rival the highest average levels of CORT observed in amphibians in response to stressors (Coddington and Cree, 1995; Gobbetti and Zerani, 1996; Hopkins et al., 1997; Jurani et al., 1973; Zerani et al., 1991). Although, increased glucocorticoid levels may have worked to rebalance plasma ion homeostasis earlier in infection, increased baseline glucocorticoid levels may also have caused some of the changes we observed in leukocyte profiles, metabolic rate, appetite, body mass, and body condition. Our results mirror those from chapter 1 of this dissertation, with infected individuals containing significantly altered leukocyte profiles, increased resting metabolic rate, decreased food intake, decreased body mass, and decreased body condition around the time when they become diseased. Appetite suppression may be the worst of the effects during infection, because of its aforementioned effects on plasma ions. With little cutaneous and digestive ion inputs, plasma ion levels eventually become unsustainable and death follows.

The time course that we present does not follow our prediction that CORT levels would increase soon after sodium levels decreased. Sampling individuals at time points following 39 days post infection could identify when CORT levels significantly increase. As plasma ions began to decrease, we observed data points that suggested that elevated CORT values may act to rebalance plasma ion homeostasis. Future studies that tease apart the precise mechanisms of chytridiomycosis pathogenesis may help researchers treat *Bd*-infected frogs as well as contribute to understanding why there is such large variation in susceptibility to chytridiomycosis among amphibian species. Given the devastating effects chytridiomycosis is having on amphibian populations, understanding these mechanisms may prove essential to conservation of amphibians in the future.

Figures:

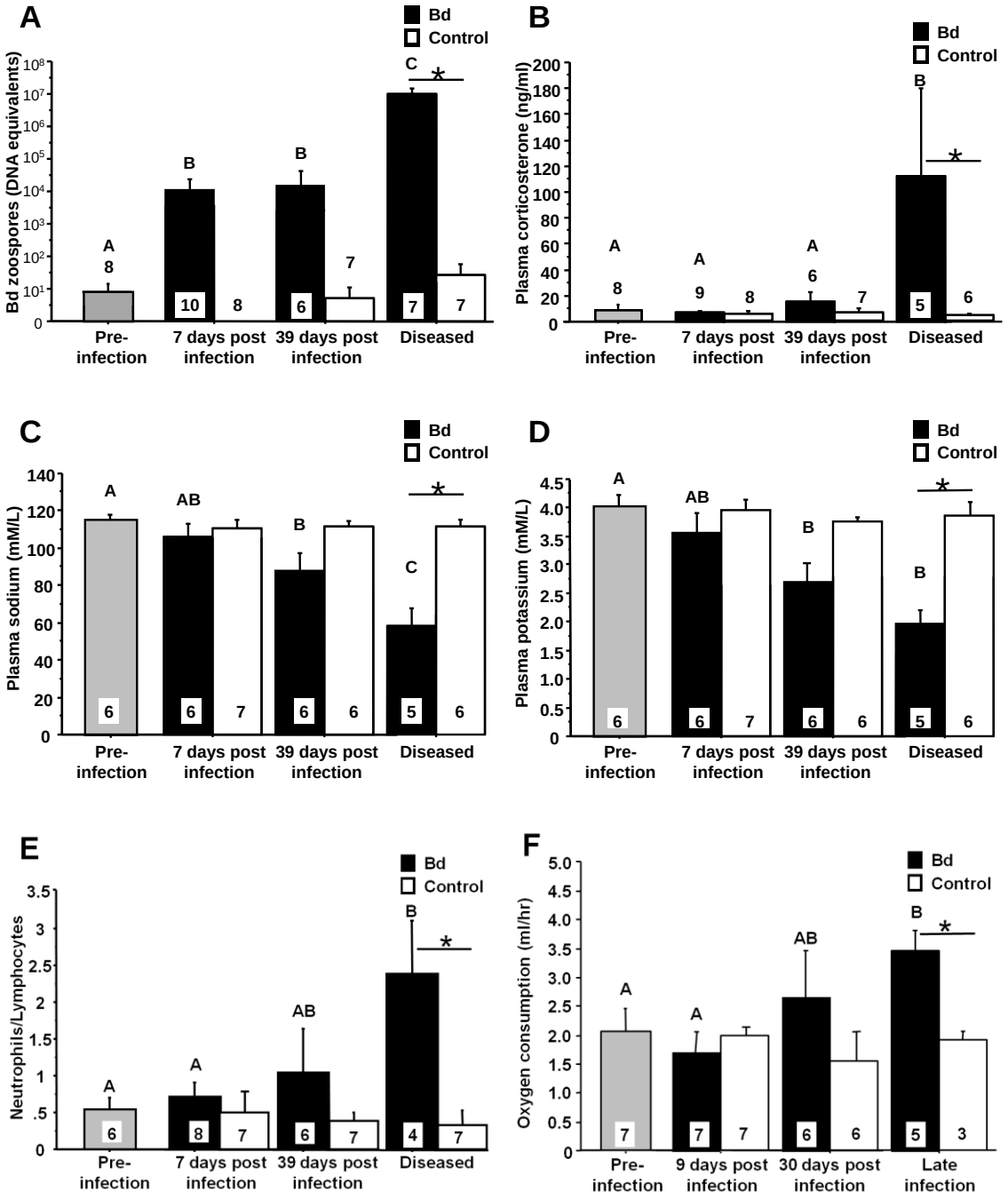

Fig. 2.1. Average (A) *Bd* burden, (B) baseline plasma corticosterone, (C) plasma sodium, (D) plasma potassium, (E) Neutrophil-lymphocyte ratio, and (F) oxygen consumption of *Litoria caerulea* either infected with *Batrachochytrium dendrobatidis (Bd)* or uninfected controls

assessed prior to and throughout infection (+SEM). Values reported at the "Diseased" time point were assessed when infected frogs displayed clinical signs of disease (Range: 32-88 days post infection). Values reported at the "Late Infection" time point were assessed an average of six days prior to when infected frogs displayed clinical signs of disease. Significant differences among time points for infected frogs are denoted by upper case letters. Significant differences between treatments at the Diseased/Late infection timepoints are denoted by an asterisk. Numbers inside bars denote sample sizes.

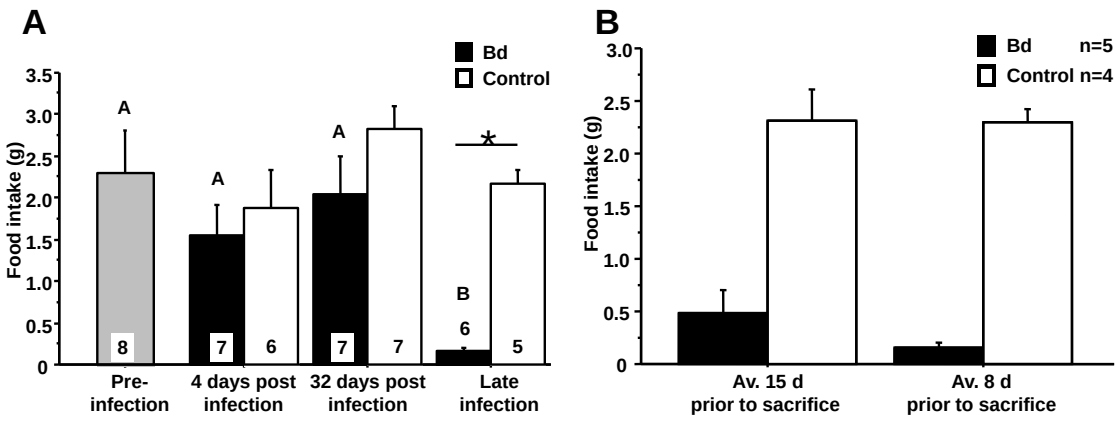

Fig. 2.2. Average food intake of *Litoria caerulea* infected with *Batrachochytrium dendrobatidis* or uninfected control frogs assessed either (A) prior to and throughout infection or (B) repeatedly within an average of 15 days prior to when infected individuals displayed signs of disease (+SEM). Values reported at the "Late infection" time point were assessed an average of seven days prior to when infected frogs displayed clinical signs of disease. Significant differences among time points for infected frogs are denoted by upper case letters. The significant difference between treatments at the "Late infection" time point is denoted by an asterisk. Numbers inside bars denote sample sizes. Repeated measures of food intake (B) revealed a significant difference between treatments within an average of 15 days prior to sacrifice.

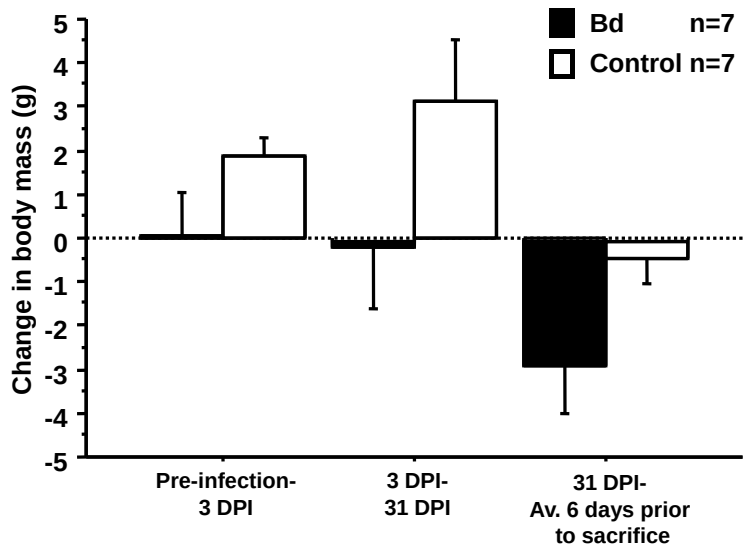

Fig. 2.3. Average change in body mass between time points prior to and throughout infection of control *Litoria caerulea* and frogs infected with *Batrachochytrium dendrobatidis (Bd).* Infected frogs lost significantly more mass than controls across time periods.

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Chapter 3. The metabolic effects of glucocorticoids in an anuran amphibian.

Abstract

Acute glucocorticoid secretion functions in freeing energy stores, increasing metabolism, and reducing energy consuming processes (e.g. reproduction and cellular immunity). Although glucocorticoids have been documented to increase whole animal metabolism in several vertebrate groups, only one study has documented this effect in amphibians, and no study has documented this effect in anurans. Given recent global population declines, understanding how amphibians respond to environmental stressors has become more urgent. Many factors linked to declines cause increase corticosterone (CORT; the primary glucocorticoid in amphibians). For example, in chapters one and two of this dissertation, we documented that infection with the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, increases both plasma CORT and resting metabolic rate in *Litoria caerulea*. To examine the possibility that CORT may have influenced metabolic rate in *L. caerulea* we treated individuals with exogenous corticosterone (CORT; the most abundant amphibian glucocorticoid; 400ug CORT/20ul DMSO) and observed 1.8 and 2.2-fold increases in resting metabolic rate three and six hours post injection, respectively. This treatment also caused physiologically realistic 33-fold increases in plasma glucocorticoids at similar time points. These data suggest that acute response glucocorticoid secretion is energetically costly in anuran amphibians. Considering that many factors suggested to cause amphibian population declines also elevate glucocorticoids, we suggest that the metabolic cost of glucocorticoids be considered in future amphibian decline discussions.

1. Introduction

The vertebrate stress response is a suite of physiological changes that help organisms cope with environmental perturbations (Wingfield et al., 1998). One component of the stress response is the hypothalamic-pituitary-adrenal (-interenal in amphibians) axis, which secretes glucocorticoids to modulate the organism's energetic response to a stressor. For example, glucocorticoid secretion causes gluconeogenesis, downregulation of energetically costly but not immediately relevant processes (e.g. reproduction and cellular immunity), and increases in whole organism metabolic rate (Sapolsky et al., 2000). While this response is thought to be adaptive in the short-term, it may be maladaptive in the long-term (Sapolsky et al., 2000).

Glucocorticoids have been highlighted for their potential use as a biomarker in conservation studies (Busch and Hayward, 2009). Amphibians are in dire need of conservation, given recent global amphibian population declines (Stuart et al., 2004); however, little is known about the connection between glucocorticoids, metabolism, and how these evolutionarily conserved physiological responses may, under new environmental challenges, contribute to population dynamics (Wack et al., 2012; Warne et al., 2011). Several factors suggested to contribute to amphibian population declines have been shown to influence glucocorticoid secretion [e.g. anthropogenic contaminants (Gendron et al., 1997; Glennemeier and Denver, 2001; Goulet and Hontela, 2003; Hayes et al., 2006; Hopkins et al., 1997; Hopkins et al., 1999; Larson et al., 1998; Peterson et al., 2009; Ward and Mendonça, 2006), disease (Belden and Kiesecker, 2005; Warne et al., 2011), habitat alteration (Denver, 1998; Newcomb Homan et al., 2003), and predation (Denver, 2009; Fraker et al., 2009)]. In contrast, only one study has documented the effect of glucocorticoids on metabolic rate. Wack et al. (2012) administered exogenous corticosterone (CORT, the most abundant glucocorticoid in amphibians) to

salamanders, *Plethodon shermani*, raising plasma CORT to physiologically relevant levels, and observed a significant increase in metabolic rate; however, data are still needed for anuran amphibians. Additionally, few studies have documented the link between decline factors, glucocorticoids, and metabolism. In chapters 1 and 2 of this dissertation, we observed that frogs, *Litoria caerulea*, infected with the fungus, *Batrachochytrium dendrobatidis* (which has been linked to amphibian declines the world-over), experience significant increases in resting metabolic rate and baseline plasma glucocorticoids; however, it is unclear whether glucocorticoid secretion caused the metabolic changes.

The goal of our study is to determine if CORT influences resting metabolic rate in *L. caerulea*, which would be the first time this relationship has been documented in an anuran amphibian. We treated *L. caerulea* with exogenous glucocorticoids and documented effects on plasma corticosterone and resting metabolic rate at several time points post treatment.

2. Methods

2.1 Animal care

Litoria caerulea were obtained commercially (Tri Reptile, Miami, FL) in autumn 2011. Frogs were recently collected from the wild in Indonesia. Animals were acclimated to lab conditions for at least one month prior to experimental manipulation. Amphibians were housed individually in plastic containers (17 cm x 17 cm x 17 cm) containing paper towels saturated with well water. Wet paper towels were replaced twice a week for the duration of the study. Once a week animals were fed approximately 10% of their body weight in 2.5 week old crickets coated in multi-vitamin dust. Any crickets not consumed were removed after 24 hours. Light was provided by full spectrum light bulbs on a 12:12 light/dark cycle.

2.2 Experimental design and treatments

The effects of glucocorticoid treatment on resting metabolic rate (RMR) and plasma glucocorticoids were determined in two separate experiments. In experiment 1, frogs were separated into two treatment groups: vehicle control (intraperitoneal injection of 20 µl DMSO, n=10) or CORT (intraperitoneal injection of 400 µg CORT in 20µl DMSO, n=13). Metabolic rate is often correlated with body mass. To minimize this effect on treatments, individuals were allocated to treatment groups such that treatments had similar masses and standard errors for body mass. Individuals were sampled repeatedly for RMR. A preliminary experiment determined that sampling individual's metabolic rates repeatedly within one day had an effect on baseline oxygen consumption, so instead, non-injected baseline RMR as well as RMR three, six, and 24 hours post injection were determined at least a month apart. Once individuals were injected with vehicle or CORT and sampled for RMR, they were not injected and sampled again for at least 30 days to reduce the effects of the previous injection and sampling. Both treatment and time were treated as fixed effects.

Experiment 2 was conducted 33 days after the conclusion of experiment 1 to reduce the effects of previous treatment. Individuals from previous treatment groups were evenly divided among three new treatment groups: vehicle control, injected intraperitoneally three hours prior to bleed (Control, 20 µl DMSO, n=6); CORT injected intraperitoneally three hours prior to bleed (CORT 3 h post injection, 400 µg in 20µl DMSO, n=7); and CORT injected intraperitoneally six hours prior to bleed (CORT 6 h post injection, 400 µg in 20µl DMSO, n=6). Frogs were again allocated to treatment groups such that treatments had similar masses and standard errors for body mass, to reduce the effect of body mass on treatments. A randomly chosen individual was injected every 15 minutes within each treatment group (CORT-treated individuals sampled 6 h post injection were treated between 900 and 1030, CORT-treated individuals sampled 3 h post

injection were treated between 1155 and 1335, and vehicle-treated control individuals sampled three hours post injection were treated between 1150 and 1320). To minimize time of day effects, all individuals were bled within a 105 minute period (1450 and 1635).

2.3 Respirometry

Closed system respirometry was used to measure resting metabolic rate (oxygen consumption) following the methods of Ward et al. (2006) with the following changes. Frogs were acclimated in 140 ml syringes (Monoject, Sherwood Medical Industries, Ballymoney, UK) for at least 45 minutes in a darkened incubator (25°C). Frogs were incubated for ~50 minutes in a darkened incubator (25°C). Any frogs that urinated or defecated during incubation were excluded from analyses. Frogs fasted for at least 5 d prior to being sampled. Frogs were weighed following respirometry. Body masses of frogs ranged from 14-40g.

2.4 Blood collection and radioimmunoassay

Blood was collected directly from the heart following double pithing. Blood was then centrifuged for 4 minutes at 3,500 x g and the plasma was frozen and stored at -20º C for later use in corticosterone radioimmunoassay. Plasma corticosterone concentrations were determined by radioimmunoassay as described by Mendonça et al. (1996). All samples were run in one assay. Extraction efficiency was 67% and intraassay variation was 32%.

2.5 Statistical analysis

Resting metabolic rate was compared between treatments and over time using a repeated measures analysis of variance (repeated measures ANOVA). Since each frog was given the same concentration of CORT, regardless of body mass, plasma corticosterone levels were compared among treatments using an ANCOVA with treatment as the independent variable, CORT as the dependent variable, and body mass as the covariate. Fisher's protected least

significant difference (Fisher's PLSD) tests were used for post-hoc comparisons among treatment groups for experiment 2. All statistical analyses were conducted using StatView for Windows (SAS institute, version 5.0.1).

3. Results

3.1 Experiment 1

CORT-treated frogs consumed significantly more oxygen than vehicle-injected controls (Repeated measures ANOVA; Treatment: $P=0.013$, $F_{1,16}=7.74$; Time: $P<0.001$, $F_{3,48}=7.65$; Treatment x Time: $P=0.004$, $F_{3,48}=5.13$). Frogs treated with CORT experienced a significant 1.8 and 2.2 fold increase in oxygen consumption at three and six hours post injection, respectively, compared to controls. At 24 hours oxygen consumption returned to levels similar to concurrently sampled vehicle controls.

3.2 Experiment 2

The plasma of CORT-treated frogs contained significantly more CORT at three and six hours post injection compared to vehicle-treated controls (ANCOVA, Treatment: $P<0.001$, $F_{2,15}=38.59$; Body mass: $P=0.8$, $F_{1,15}=0.09$; Fisher's PLSDs, $P<0.001$). There was no treatment by body mass interaction ($P=0.7$). CORT-treated individuals experienced a 33 fold increase in CORT at both three and six hours post injection compared to vehicle-treated controls.

4. Discussion

This is the first study to document that glucocorticoids increase metabolic rate in an anuran amphibian, as well as the second study to document this effect in amphibians. Wack et al. (2012) treated the terrestrial salamander, *Plethodon shermani*, with CORT and observed that treatment caused a significant increase in plasma CORT at four hours post injection, but not at two and ten hours post injection, as well as an increase in oxygen consumption between two and

four hours post treatment, but no effect thereafter. Differences between the Wack et al. (2012) experiments and ours preclude detailed comparisons, but the same general trend was observed. We observed that in the anuran, *Litoria caerulea*, CORT treatment caused significant increases in plasma CORT at three and six hours post treatment, as well as significant increases in metabolic rate at three and six hours post treatment, which returned to control levels at 24 hours post injection.

The CORT treatment that we used caused increases in plasma CORT that are physiologically realistic for *L. caerulea* and similar species. For example, acute captivity stress caused a maximum average increase of 13.8 ng/ml plasma CORT in *Litoria ewingi* (Coddington and Cree, 1995). Chronic infection with the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, caused increases in plasma CORT to averages of 104 and 111 ng/ml in *Litoria caerulea* (see Chapters 1 and 2 of this dissertation). In general, acute stressor-induced increases in plasma CORT range from 11-56 ng/ml in frogs (Coddington and Cree, 1995; Jurani et al., 1973). In the current study, treatment with CORT increased plasma CORT concentrations to 30 ng/ml at both three and six hours post treatment. Our study is one of the few studies of vertebrates that have observed the metabolic effects of exogenous glucocorticoids which increase plasma glucocorticoids to physiologically realistic levels (Buttemer et al., 1991; Davis and Schreck, 1997; DuRant et al., 2008; Hissa et al., 1980; Morgan and Iwama, 1996; Wack et al., 2012).

Our findings, along with other recent findings, suggest that elevations in plasma glucocorticoids are energetically costly in amphibians (Wack et al., 2012). In the current study, frogs treated with CORT experienced 1.8 and 2.2 fold increases in metabolic rate at three and six hours post treatment compared to controls sampled concurrently. The cost of these increases

becomes more apparent when we convert our values to energetic cost (1 ml of oxygen consumed = 20.1 J; Feder and Burggren, 1992). The cost of BMR (J/h) at three and six hours post injection with CORT increased by 150% and 190%, respectively, compared to baseline levels. Even if BMR had only increased for a three hour period, the extra cost would represent 17% the daily requirements to maintain BMR (0.037 kJ). Although we only observed the acute metabolic effects of increased plasma CORT, chronic increases in CORT and metabolic rate could deplete energy reserves and cause weight loss. Continued weight loss could lead to wasting and death and, thus, contribute to amphibian population declines. Several studies provide evidence that CORT induced weight loss may contribute to amphibian population declines, though they do not determine whether weight loss is mediated by elevation of metabolic rate (Denver, 1998; Hayes et al., 2006; Peterson et al., 2009; Warne et al., 2011). To our knowledge, the first two chapters of this dissertation are the first to document such effects of a decline factor on CORT, body mass, and metabolism. In these studies, chronic infection with *Batrachochytrium dendrobatidis*, a cause of amphibian population declines the world-over, increased plasma CORT and metabolic rate more than three and 15 times higher, respectively, than the values observed in the current study in the same species. Infection also caused significant decreases in body mass and body condition prior to death. It is important to note that the authors of these studies did not determine if CORT caused the effects on metabolic rate, weight loss, and body condition; however, our study suggests that it is physiologically possible in this species.

We believe that more studies should focus on the contribution of alterations in CORT and metabolism to amphibian population decline. Our data suggest that even an acute, CORT-induced increase in RMR is costly. Chronic elevation of CORT and RMR may deplete energy stores and induce wasting in declining populations trying to cope with decline factors. The

metabolic cost of CORT secretion has only recently been identified in amphibians. Authors suggest that CORT may influence amphibian population dynamics by directly modulating reproduction and immunity, but we suggest that CORTs influence on metabolism also be considered. It may directly contribute to declines by inducing wasting, but may also indirectly modulate reproduction and immunity by depleting the energy stores that they, and many other energetically expensive processes, require.

Figures:

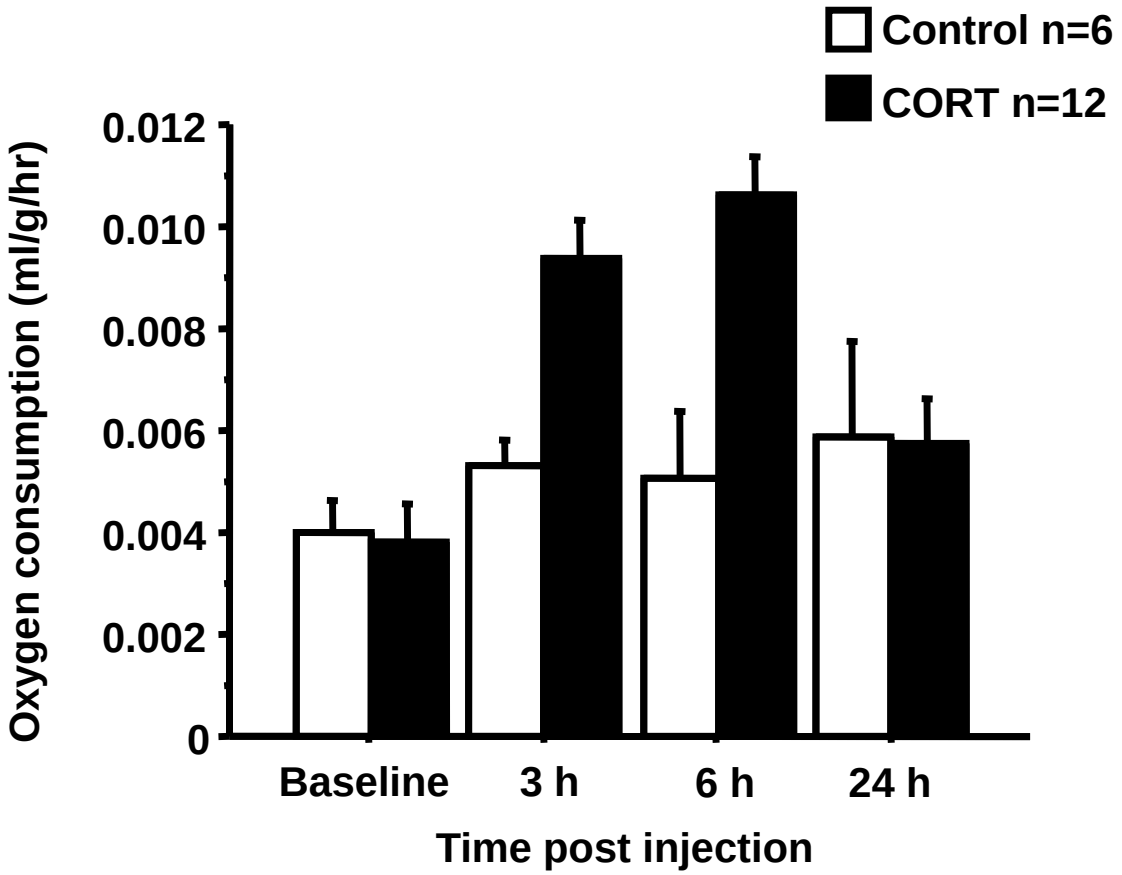

Fig. 3.1. Baseline average resting metabolic rates (+SEM) and rates 3, 6, and 24 hours post injection injected with either corticosterone (CORT; 400 μg CORT in 20μl DMSO) or vehicle (Control; 20μl DMSO) of *Litoria caerulea*. CORT-treated frogs consumed significantly more oxygen than vehicle-injected controls (Repeated measures ANOVA; Treatment: p=0.01, F=7.74; Time: p=0.0003, F=7.65; Treatment x Time: p=0.004, F=5.13).

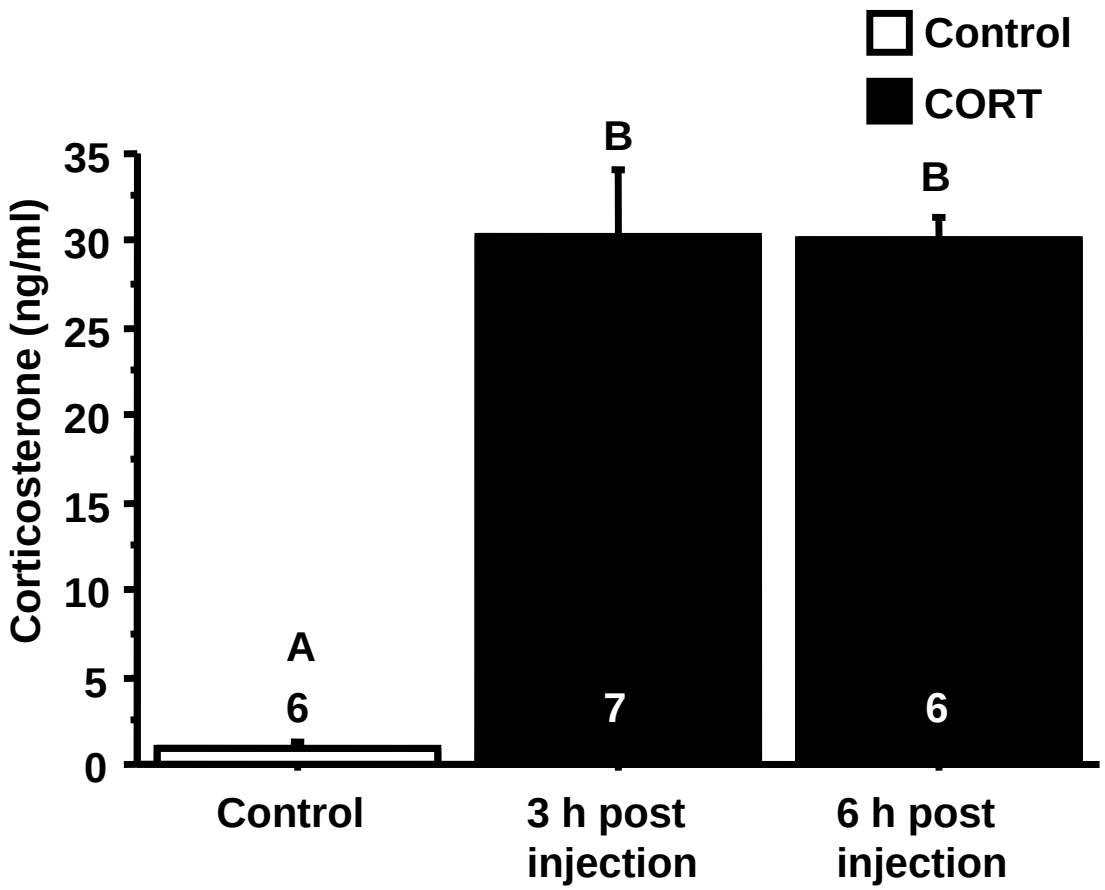

Fig. 3.2. Average plasma corticosterone (+SEM) of *Litoria caerulea* three hours post injection with vehicle (Control; 20µl DMSO), three hours post injection with corticosterone (CORT; 400 µg CORT in 20µl DMSO) and six hours post injection with corticosterone. Treatments were significantly different (ANOVA, $p<0.0001$, $F=41.31$). Different capital letters denote significant differences among treatments (Fisher's PLSDs, $p<0.0001$).

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Conclusion

The experiments contained herein are among the few experiments to document how infectious disease influences stress physiology in wild vertebrates. These experiments are also among the first to observe how disease-induced increases in glucocorticoids potentially influence variables normally influenced by glucocorticoids in non-diseased animals (Warne et al., 2011). Although we could not determine whether glucocorticoids caused the changes in these variables, significant increases in CORT were temporally associated with effects typically caused by glucocorticoids in vertebrates in non-disease situations. Specifically, individuals displaying clinical signs of disease contained significantly elevated CORT, elevated neutrophils, suppressed lymphocytes and eosinophils, and elevated standard metabolic rate.

These experiments provide novel contributions toward understanding the pathogenesis of chytridiomycosis. They suggest that plasma ion suppression, appetite suppression, increased metabolic rate, weight loss, and increased shed skin frequency occur prior to when individuals display clinical signs of disease (i.e. lethargy, loss of righting reflex, and tremors). Changes in plasma ions preceded changes in all other variables monitored in this study, including appetite, which was previously suggested to be the earliest effect of *Bd* infection (Nichols et al., 2001). This finding suggests that initial decreases in plasma ions are not due to reduced digestive sodium uptake. Instead, early plasma ion loss is likely due to disruption of cutaneous sodium inputs by *Bd* infection of the skin (Voyles et al., 2009). Once frogs display clinical signs of disease, further suppression of plasma ions occurs. This further decrease in ions can be attributed to both continued disruption of cutaneous sodium uptake and suppressed digestive

uptake due to loss of appetite. Loss of appetite and increased metabolic rate in the week prior to displaying clinical signs of disease likely contribute to observed weight loss and decreased body condition. Loss of appetite may also explain why shed skin was found more often within bins of frogs that eventually displayed clinical signs of disease, considering that many frogs consume their shed skin (Feder and Burggren, 1992). Besides disruptions to plasma ions, frogs displaying clinical signs of disease also experienced elevated plasma CORT, reduced body condition, elevated neutrophils, suppressed lymphocytes and eosinophils, as well as continued elevation of standard metabolic rate.

CORT likely mediates some of the effects observed during *Bd* infection. Previous research on amphibians suggests that hyponatremia causes increased secretion of glucocorticoids and exogenous glucocorticoids can correct hyponatremia to homeostatic concentrations (Heney and Stiffler, 1983; Stiffler et al., 1986). In Chapter 2, significant decreases in plasma sodium at 39 days post infection may have not been low enough to trigger a CORT response in all individuals. In fact, at 39 days post infection, two infected individuals contained CORT concentrations that mirror stressor-induced levels in similar species (Coddington and Cree, 1995), as well as sodium and potassium levels that parallel levels of control levels, suggesting that CORT secretion may initially help maintain sodium balance. These individuals also experienced changes in NL ratios equivalent to those observed in frogs undergoing an acute stress response (Belden and Kiesecker, 2005; Bennett et al., 1972; Bennett and Harbottle, 1968; Coddington and Cree, 1995; Davis and Maerz, 2010; Garrido et al., 1987; Jurani et al., 1973). Due to limitation in experimental design, CORT was not assessed again until frogs displayed clinical signs of disease. Future studies should sample for CORT when frogs experience significant changes in plasma sodium, appetite, or the period between 39 days post infection and

the first clinical signs of disease. By the time frogs display clinical signs of disease, they have experienced another significant decrease in plasma ions as well as a significant increase in plasma CORT. These individuals also experience changes in WBC profiles and metabolic rate that parallel the effects of CORT on these variables in non-diseased animals. Future studies should further test whether *Bd*-induced decreases in plasma ions induce a stress response that exacerbates pathogenesis.

Finally, these experiments highlight the fact that there is a lack of data on how glucocorticoids influence physiology in amphibians. For example, there is a lack of data on how glucocorticoids influence metabolic rate and appetite in amphibians (Carr et al., 2002; Crespi et al., 2004; Wack et al., 2012). Chapters 1 and 2 provide correlative data to suggest that CORT may influence these factors. Chapter 3 provides empirical data that suggests that physiologically relevant increases in plasma CORT by exogenous CORT treatment significantly elevate standard metabolic rate, but controlled laboratory data on food intake are needed to complement chapters 1 and 2. Understanding how amphibians respond to environmental change has become more urgent given recent amphibian population declines (Stuart et al., 2004). Several factors suggested to contribute to amphibian population declines have been shown to influence glucocorticoid secretion [e.g. anthropogenic contaminants (Gendron et al., 1997; Glennemeier and Denver, 2001; Goulet and Hontela, 2003; Hayes et al., 2006; Hopkins et al., 1997; Hopkins et al., 1999; Larson et al., 1998; Peterson et al., 2009; Ward and Mendonça, 2006), disease (Belden and Kiesecker, 2005; Warne et al., 2011), habitat alteration (Denver, 1998; Newcomb Homan et al., 2003), and predation (Denver, 2009; Fraker et al., 2009)]. Though the influence of anthropogenic contaminants on the stress axis has been relatively well studied in amphibians far less is known about how disease, habitat destruction, invasive predators, and climate change may

influence stress physiology. Given the powerful and far reaching effects of glucocorticoids on wildlife life histories and the contribution of environmental perturbations to biodiversity loss, understanding how these hormones mediate the interplay between environmental perturbations and life histories is essential to future conservation efforts.

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