IMPACT OF HEALTH, HUSBANDRY, AND CONSERVATION RESEARCH ON
GLUCOCORTICOID CONCENTRATIONS IN *ATELOPUS* SPECIES

by

SHAWNA CIKANEK

B.S., Kansas State University, 2011

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Clinical Sciences
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2013

Approved by:

Major Professor
James W. Carpenter, MS, DVM, DACZM

Abstract

In many species, temporary increases in glucocorticoids (GC) can be used to identify changes in adrenal activity in response to acute stressors. For this research, GC metabolites were identified in fecal extracts from various *Atelopus* species. The objectives were to identify possible correlates between GCs and health status, assess the impact of husbandry protocols on adrenal activity, and evaluate the sub-lethal effects of antifungal bacteria used for protection of frogs against the chytrid fungus (*Batrachochytrium dendrobatidis*; *Bd*).

The first study examined whether fecal GC concentrations can be correlated with animal health and behavior changes in a captive setting. *Atelopus zeteki* with varying degrees of dermatitis were categorized based on the severity of their skin abnormalities and GC metabolite concentrations were analyzed to detect correlations between severity of disease and GC metabolite concentrations. Similarly, behaviors that may indicate elevated stress levels (e.g., time spent in hide) were analyzed to detect correlation between disease and behavior changes. There was no correlation between fecal GC metabolites and health status of the animal or between health status and amount of time spent in hide.

The second study established ex situ colonies of two Panamanian frog species, *Atelopus certus* and *Atelopus glyphus*, to determine how male group size affects behavior and GC levels. When housed in groups of eight, animals initially had elevated GC concentrations and interacted aggressively, but these instances declined substantially in the first 2 weeks of being housed together. Thus, captive *Atelopus* populations can be housed in same-sex enclosures without causing sub-lethal stress on the individuals involved.

The third study examined the ability of antifungal bacterium from Central America to propagate on *Atelopus* skin as a preventative treatment for *Bd* and the sub-lethal effects of each bacteria species on adrenal function based on GC analysis. Four species of bacteria (*Pseudomonas* sp., *Pseudomonas putida, Chryseobacterium indolgenes,* and *Stenotrophomonas maltophili*) were found to be successful *Bd* inhibitors in vitro. There were no detectable effects of bacterial exposure with GC metabolite concentrations over time for any of the treatments assessed.

Table of Contents

List of Figures

List of Tables

Acknowledgements

I would like to express my gratitude towards the people who have helped and supported me throughout the topsy-turvy experience of fulfilling my master's requirements. To all of my friends and colleagues at the Smithsonian Conservation Biology Institute and on the east coast; Geoff Reynolds, Warren Lynch, Sarah Putman, Matt Becker, and Dr. Marc Valitutto, thank you all for the invaluable training and mentorship that you provided while I was at the Smithsonian. You were my guides when I was learning the ropes and some of the fondest memories I have from my master's experience revolve around our everyday interactions. Thank you for making my master's experience worthwhile.

Dr. Janine Brown, Dr. Brian Gratwicke, Dr. David Grieger, and Dr. Katharine Hope you all were some of my most influential mentors throughout my master's experience. Without allowing me to call you about any little concern or being willing to edit my papers at all hours of the night, I would not have ended up with a product I am passionate about and proud to sign my name on. All of you cared not only about my thesis but the journey I took to create the finished product. On top of that, you were all so kind and made me feel welcome every time we spoke. I feel incredibly blessed to have had the opportunity to work with each of you and I hope you realize the impact you have had on me and my career.

A special thanks must go out to my "stellar" primary advisor, Dr. James W. Carpenter. Why you took a chance on the naïve undergraduate who barged into your office and requested a chance to do her master's under your tutelage, I'll never know but I will forever be grateful. My entire master's experience was made possible because you believed that I could do it all. You set the bar high from day one and never once held me to a standard lower than I was capable of. I hope in all our future endeavors you continue to push me to be better and help guide me to become the best possible version of my professional self.

Chapter 1 - Introduction

1.1 Amphibian Biology

Amphibian comes from the Greek words "amphis" meaning double and "phios" meaning life to represent the multiple amphibian life cycles that take place in both water and land [1]. The class Amphibia is composed of three orders. The largest of the three is Anura and is made up of frogs and toads. The other classes are Caudata and Gymnophionia and represent salamanders/newts and caecilians, respectively [1]. Amphibians are ectotherms that obtain their internal temperature from external sources such as air, water, and substrate [2,3]. Amphibian skin is highly permeable and more integral to homeostasis than in other species. Frogs interact with the environment through skin via respiration, water balance and thermoregulation [4–6]. Other skin functions include anti-predator toxins and anti-fungal defense [7]. The skin is made up of two layers: an inner dermal layer and an outer epidermal layer that lacks conventional protection (i.e., hair, scales, feathers) [6]. Some species have granular (poison) glands located in the dermis that protect from bacterial and fungal infections by releasing anti-microbial peptides [8]. In frogs, these granular glands are most prevalent on the dorsal surface [4]. Panamanian golden frogs (*Atelopus zeteki*) in particular produce a potent zetekitoxin that blocks the sodium channels of their predators [9].

Amphibians do not drink water; they absorb it from substrates in the environment [3]. They breathe using a mixture of gaseous exchange across the skin surface and air pumped through the lungs by raising and lowering of the throat [4]. For both respiration and breathing, the surface of the skin must be moist. Mucous glands are found on the epidermal layer of the skin and help the skin remain moist while also aiding in thermoregulation [7]. Amphibian skin has two separate layers of chromatophores that allow them to change color in response to environmental conditions [10]. Activation of chromatophores under stressful conditions such as excess handling, chemical irritants in water, inappropriate temperature range, or bacterial or fungal infections causes a physical presentation of environmental disturbance, such as erythema, to occur [5,6]. All of the above functions of amphibian skin make them highly sensitive to environmental conditions and susceptible to injury and disease [5]. Husbandry practices are of paramount importance in the management of captive amphibians because so much of an animal's

health is based on its environment [3]. Temperature, humidity, and water quality all have a direct impact on amphibian health [6].

Amphibians are able to tolerate a wide range of habitats and thrive in almost every location on earth. Despite the broad territory, amphibians cannot tolerate sudden environmental changes and their highly permeable skin leaves them susceptible to disease. Thus, the unique function of amphibian skin is not only a key factor in their survival, but also a point of vulnerability.

1.2 Stress, Homeostasis, and Allostasis

The concept of stress began in the early 1930s and has since been a source of debate among scientists. Stress is an indicator of an animal's wellbeing and can be used to indicate overall health [11,12]. The word stress is widely used and loosely defined and, as a result, there is currently no standard definition of stress. One definition of stress is a stimulus that requires an immediate energetic response, while another is that it is simply a disruption in homeostasis [12–14]. By other definitions, stress must elicit endocrine and behavioral coping mechanisms [15]. It is generally accepted that the word stress is used in three different ways: as an event, a response to a stressor, or a state of being [13,16]. Terms have been devised to differentiate among the three meanings: stress is a state in which homeostasis is lost, a stressor is any factor that causes a disruption in equilibrium, and a stress response is a trigger of physiological and behavioral mechanisms that restores homeostasis [12,14,17]. However, misunderstanding also surrounds the term homeostasis, which is often used when explaining stress. Homeostasis is generally defined as the stability of physiological and behavioral mechanisms through change [13,18,19]; however, the term may not adequately incorporate all processes involved in an animal adjusting to a stressor [20]. For example, the concept of homeostasis and stress only includes physiological and behavioral changes [20,21] but does not address the impact that genes and prior experiences can have on health, disease and the ability of an individual to cope with environmental disruptions [13].

The alleged inadequacies surrounding the words "stress" and "homeostasis" have led to the development of the concept of allostasis which includes the terms allostasis, allostatic load, and allostatic overload [13]. Allostasis was designed to include everyday factors such as social organization, food intake, and metabolic demands to the general description of homeostasis [13].

Allostasis is defined as maintaining stability through change, allostatic load is the cumulative impact of physiological coping mechanisms, and allostatic overload is a state in which there is a cost to the body as an individual tries to adjust to a change in the environment [15,18,19]. The concept of allostasis is built on the idea that life is broken up into multiple stages. Events such as breeding, parturition, or an environmental perturbation make up a continuum that determines how an animal will cope [13]. In this model, the word "stress" is an event that is restricted to environmental changes that lead to allostatic load [15]. This system is based on the idea that an individual animal determines whether an event is a stressor based on prior experience and the events that trigger a stress response can vary over time (i.e., an amphibian becomes accustomed to handling and thus no longer mounts a physiological response to being held) [14,22]. The idea of allostatic load relies heavily on an animal's energy demands in which energy usage is thought of as a fluid requirement that fluctuates with different life stages [19]. If an animal has an increase in the amount of energy required to maintain homeostasis then there will be a subsequent rise in allostatic load [17]. Two types of allostatic load have been identified in the concept of allostasis. The first occurs when the demand of energy on an individual's body exceeds the energy available. The second is characterized by an allostatic state in which an animal eats an overabundance of food for a prolonged period of time [18,19]. The concept of allostasis differs from the idea of stress because it incorporates the metabolic demands of normal life stages as well as those caused by unpredictable environmental changes [13]. This framework allows for an animal's individual experiences such as social status, changes in the environment, and health to redefine the classical concept of homeostasis [19].

The concept of allostasis, while more specific, is also flawed. An extension of the allostasis concept, called the reactive scope model, was proposed to address the ambiguity of energy expenditure and input [16]. This new model delves deeper into the definition of homeostasis and includes changes in behavior, central nervous system and cardiovascular function, as well as mediators of immune function. The only parameters that were included in this thesis were the monitoring of stress related hormones and how they relate to animal behavior and health. Because of this, the standard definitions of stress, stress response, and homeostasis as defined above, are adequate for this thesis and the concept of allostasis and the reactive scope model will not be incorporated.

1.3 Stress Response

The stress response is an important physiological event that allows an animal to react appropriately to a stressor. It relies on the activation of the hypothalamic-pituitary-adrenal (HPA) axis, beginning with the release of corticotropin-releasing hormone (CRH) in the hypothalamus of the brain and ends with the release of one of two glucocorticoid (GC) steroid hormones: cortisol or corticosterone that cause physical and behavioral changes [22,23]. The amount of GC produced depends on the intensity of the stressor; a more intense stressor means more GCs will be released [12].

The two most important physiological responses to stress are the stimulation of the sympathetic nervous system (SNS) and the activation of the HPA axis [14]. Both are activated by the central nervous system. An animal responds to a stressor using three steps known as the general adaptation syndrome. The first step is an alarm phase in which the SNS is activated. During the second, resistance phase, the HPA axis is stimulated. Finally, the exhaustion phase occurs when the elevated GCs begin to have a deleterious effect [14].

The HPA axis consists of hypothalamic paraventricular nucleus (PVN), anterior pituitary gland, and adrenal cortex. Under normal circumstances, the hippocampus inhibits the HPA axis. Immediately upon the detection of a stressor, the SNS causes the adrenal medulla to release the catecholamines norepinephrine and epinephrine into the vascular system. Simultaneously, the PVN of the hypothalamus releases CRH into the portal system that connects the hypothalamus and anterior pituitary. This causes the anterior pituitary to release adrenocorticotropic hormone (ACTH) into the blood stream and, within minutes, the adrenal cortex releases GCs above basal level. This entire pathway is controlled by a negative feedback loop. When the concentration of ACTH is elevated, it is detected by ACTH receptors in the brain to suppress the initial steps of the HPA axis [12,24,25]. Fine control of HPA axis activation is critical because the inability to terminate stress induced HPA activation can result in chronic stress, which has negative health effects [22,26]. Glucocorticoids are produced not only in response to negative events but also at the basal level to control normal homeostatic activity. They increase energy by means of increased gluconeogenesis, and decrease sensitivity to insulin and protein and fat metabolism [14]. They also increase cardiovascular tone, regulate the immune system, and inhibit digestion [18]. Altogether, the stress response allows an animal to properly react to acute changes in homeostasis that constitutes a typical stressor [27].

1.4 Non-Invasive Hormone Monitoring

Measuring adrenal stress hormones as indicators of physiological stress in captive and wild vertebrates continues to grow in popularity. Glucocorticoids (GCs) are monitored because they are stable steroid hormones that can be measured for both field and lab research [14]. The two GC hormones commonly used as indicators of stress are the steroid hormones corticosterone and cortisol, depending on the species. It is generally accepted that most mammals and all fish produce mainly cortisol whereas birds, reptiles, and amphibians produce mainly corticosterone [12,22,23,28]. Not all studies use the GC that is most commonly monitored in a particular species. This study has proven the validity of monitoring cortisol rather than corticosterone in amphibians (See Appendix B). Glucocorticoids can be measured in samples such as urine, feces, blood, hair, and feathers [24], and there are advantages and disadvantages to each approach. The most common method of evaluating stress hormones is blood sampling [29–31]. A large portion of the total GCs are bound in the blood to a plasma protein called corticosterone binding protein. This protein is too large to leave a capillary unassisted so GCs remain in circulation thus allowing blood sampling to provide an immediate snap-shot of the hormone profile of an animal [11]. Unfortunately, blood sampling generally cannot be achieved without handling and/or restraining an animal [32,33] which in itself can cause artificially high GC concentrations [34–36].

Non-invasive hormone monitoring has grown in popularity as a way to avoid influencing GC results. The use of hair and feathers is an excellent way to monitor chronic stress but a poor way to determine short term changes in stress hormones [24]. A rising awareness of the validity of monitoring GCs in excreta has led to an increase in noninvasive methods using urine and feces because samples can be obtained without disturbing the animal [37]. While urine is easily attainable and can be collected on a regular basis, it is not possible to obtain urine samples in all situations [24]. For example, urine samples from animals living in an aquatic environment could potentially be diluted by water. In contrast, fecal samples can be easily collected and have been used to successfully determine GC concentrations in aquatic mammals [38]. Fecal glucocorticoid metabolites (FGM) are those metabolized by the liver prior to excretion and reflect the number of unbound GCs in the blood stream [11]. Fecal glucocorticoid metabolites represent pooled quantities of GCs over time and are not as prone to fluctuations, such as normal pulsatile rhythms, because time from GC release to rise in FGM is much longer than blood

sampling [24,29]. Despite common perception, elevated FGM concentration does not always indicate an elevated level of negative stress. Activities including courtship and hunting also cause an increase in GCs yet they are not considered unduly stressful events [21]. Biological factors such as gender, season, and reproductive status must be considered when interpreting results because they can effect GC concentrations [29]. It is also important to establish a baseline concentration for a species or individual to determine what an 'elevation' in GCs actually means [37]. Caution must be used after collection because sample age, storage, and collection techniques can also skew results [37]. It is recommended to freeze fecal samples immediately after collection for best results [24].

1.5 Male-Male Interaction

Communication in anurans occurs for a variety of reasons including courtship, advertisement, and territory defense. These interactions can be in the form of physical altercations, vocalizations, or, in the case of *Atelopus* species, visual foot signaling [39,40]. Vocalization is a useful tool among amphibians because, while majority of vocalization occurs between males [41], females tend to prefer the male with the loudest call and males can use their call to space themselves out and avoid direct confrontation [4]. For years it was believed that the Panamanian golden frog (*Atelopus zeteki*) did not communicate vocally because they lack a tympanic middle ear. This notion was disproved in 1996 when a field study using playback vocalization resulted in behavioral responses to sound. It is now believed that *A. zeteki* have the capability to communicate via visual or acoustic signals yet prefer visual because they live among noisy stream beds [40].

Territoriality occurs when there is competition for a limited resource such as a mate, food, or space. Aggressive behavior related to territorial claims is well documented in amphibians [42], yet the reason frogs display territoriality is not well known [43] and may result from competition over resources like food, mates, and shelter [44,45]. The primary form of aggression in *Atelopus* species is vocalization. Types of calls include advertisement, courtship, and encounter, and, in general, males prefer to use non-physical displays to avoid direct contact with other males [4,39]. Confrontation begins with advertisement calls and foot signaling followed by territorial calls and finally, if neither animal's calls have dissuaded the opposition, physical altercation [39,45]. When males meet, vocalization continues until one of the males

6

flees or they engage in physical combat [39]. While there is little evidence of hierarchy in amphibians, the outcome of a fight is largely reliant on size [42]. Proximity and time of year play an important role in male-male aggression. For neotropical *Atelopus* species, the only instances of territoriality occurs during the wet season (late May to mid-November) because those months coincide with the breeding season [46]. In addition, frogs from highly dense populations were more aggressive than frogs from low density populations [47] indicating that habitat availability plays a large role in occurrence of aggressive interactions.

1.6 Amphibian Decline and Assurance Populations

Amphibians are disappearing around the globe at an alarming rate. They are more threatened and declining faster than either mammals or birds. According to the 2004 International Union for Conservation of Nature (IUCN) Red List, amphibians are the most threatened of any major animal group on earth [48]. Of the more than 6,000 known species of amphibians, almost half are experiencing a population decline and 52 species move one category closer to extinction each year [49,50]. Nearly 10% of the world's amphibians are considered critically endangered and this number is undoubtedly low because an estimated 25% of all species are considered data deficient and cannot be assessed [1,49].

There are varying opinions on the secondary causes of the amphibian crisis and hypotheses include habitat loss, climate change, ultraviolet B (UVB) radiation, and the fungal disease, chytridiomycosis, caused by *Batrachochytrium dendrabatidis* (*Bd*) [1,51,52]. A study in 2005 tracked 32 species of *Atelopus* that declined despite living in a protected area. Of the 32 species in protected ranges at that time, 22 disappeared completely without experiencing any habitat reduction [53] yet despite this evidence, a report in 2008 suggested that many amphibian declines were due to habitat loss [51]. Climate change is also a proposed factor for amphibian decline [53], yet not all studies reveal a clear effect of climate change on amphibian populations [52]. A novel hypothesis for amphibian loss is that global warming has led to more ultraviolet B radiation exposure which causes mutations in the DNA of frogs and lead to a weakened immune system and increased susceptibility to disease [1].

While the above-mentioned hypotheses may play a small role in the drastic amphibian population declines, researchers generally agree the main cause is *Bd* [51–53]. *Bd* was first reported in the early 1980s in Ecuador but the connection between amphibian decline and the

arrival of *Bd* was not made until much later [51,52]. Upon arrival of *Bd* in a new location, nearly 50% of amphibian species and 80% of individuals in that area will die within 6 months [54]. Neotropical amphibians are more affected because *Bd* thrives in cool, humid environments and, because of this, the Bufonidae family is declining at a faster rate than any other [49,52]. More specific to this project, of the 113 *Atelopus* species that belong to the Bufonidae family, 30 are possibly extinct and only 10 have stable populations [54]. Currently, no tools are available to control or prevent the spread of this disease in the wild, leaving the creation of captive assurance populations the only tool to save some species [55].

 Atelopus species are of high priority for rescue populations because of their increased susceptibility to the disease [53]. A stable ex situ population of *Atelopus* should consist of at least 20 males and 20 females [56]. Project Golden Frog was launched in 1999 as a proactive attempt to prevent the extinction of one of Panama's most culturally significant species, the Panamanian golden frog (*Atelopus zeteki*). Because of the lack of proper housing on site in Panama, many wild caught specimens were shipped to zoos in the United States until a facility could be built in Panama. An ex situ facility called El Valle Amphibian Conservation Center (EVACC) was built in El Valle, Panama, in 2007 to house threatened amphibians until they could be released in the wild. Despite the intention to do so, *A. zeteki* that had been exported from Panama were not returned to Central America because of the fear they might introduce foreign pathogens [51]. Further progress was made when a second ex situ facility opened in Gamboa, Panama, under the newly established Panama Amphibian Rescue and Conservation Project. Collectively, these facilities house five of the six *Atelopus* species from Panama; *A. zeteki, A. varius, A. limosus, A. certus*, and *A. glyphus*. [51]. The sixth known *Atelopus* species from Panama, *A. chiriquiensis*, has not been recorded since 1996 [52] and may be extinct. With amphibians facing more threats and challenges than ever, these rescue populations may be the difference between survival and extinction.

1.7 *Batrachochytrium dendrobatidis*

 Batrachochytrium dendrabatidis (*Bd*) comes from the Greek words "batrachos" meaning frog, and "chytra" meaning pot referring to the shape of the flask-like zoosporangia seen microscopically [57]. The word *Dendrobatidis* was chosen because the poison dart frog (*Dendrobates auratus*) was the first amphibian on which *Bd* was isolated [58].

Batrachochytrium dendrobatidis is the causative agent of the fungal disease, chytridiomycosis, the only known chytridiomycota that affects vertebrates. It is found in water and soil all over the world [1,58], but it was not until 2009 that scientists uncovered how *Bd* leads to mortality in amphibians. Originally, it was thought that *Bd* slowly depletes innate skin defenses [59], but it was later discovered that electrolyte transfer across the skin was reduced by more than half in chytrid infected individuals. By disrupting cutaneous function, chytrid eventually leads to cardiac heart failure [60]. There has been some debate as to whether *Bd* is a novel pathogen spreading into new geographical locations or an opportunistic disease that has recently become prevalent because of global warming [61,62]. The latter was disproved when an experiment involving nearly 100 frog populations and three separate basins of water did not detect *Bd* at the start of the experiment yet population infection reached 100% within 4 years [63]. The impact of *Bd* can vary significantly among amphibian populations. One study on *Rana mucosa* showed that chytrid can lead to two outcomes within the same species, causing either rapid infection and nearly 100% mortality, or a decline in population while persisting at low levels for extended periods of time [64]. Another study on *R. mucosa* monitored a population that coexisted for 6 years with the *Bd* pathogen [59] reaffirming that populations can survive with the pathogen. When low levels of *Bd* are present and the population continues to thrive, individuals can lose and regain the pathogen multiple times [64]. Because of this, it has been hypothesized that antifungal pathogen load determines the fate of a population. When *Bd* zoospore load is high (above 10,000 zoospores per individual), mass extinction can be expected within a population [63]. One proposed reason for differences in pathogen severity is that there are variations in natural microbial and antifungal peptides found on the skin of amphibians [65,66].

After discovering that amphibians can coexist with low levels of the *Bd* pathogen, it was determined that preventing infection intensities from reaching deadly amounts could be an effective way to manage the disease in the wild [63]. Some of the earlier studies on *Bd* inhibition in salamanders showed multiple genera of naturally occurring anti-chytrid bacteria present on their skin proving that a wide array of bacteria contain antifungal properties [67,68]. *Janthinobacterium lividum* was isolated from salamanders and proved to be lethal to *Bd* by producing an antifungal metabolite called violecein [69,70]. Application of *J. lividum* to *R. mucosa* proved equally as effective in controlling *Bd* [71]. It was determined that the higher the amount of *J. lividum* present on the skin of an amphibian, the higher the amount of violecein

detected suggesting that violecein is a secondary metabolite produced only when *J. lividum* densities are high [71]. This experiment proved that bacteria from one species of amphibian can be transferred to another and used to successfully combat *Bd*, leading to the idea that probiotic bacteria could be used as a means of bio augmentation to fight *Bd* in the wild.

A species of amphibian that did not respond successfully to *J. lividum* was the Panamanian golden frog [55]. In one experiment, *J. lividum* persisted on the skin of *A. zeteki* for 5 weeks until *Bd* loads increased and drastic population declines were observed. Postmortem analyses revealed that *Bd* loads were significantly lower on *J. lividum* treated individuals but the bacteria density was not high enough to prevent mortality [55]. The conclusion was drawn that *J. lividum* is not an adequate probiotic to use for *Atelopus* spp. based on the results using *A. zeteki* as a representative for the *Atelopus* genus. Thus, a follow-up experiment was designed to research additional antifungal bacterial species native to Central America where *Atelopus* spp. are commonly found. Study III of this thesis is based on this hypothesis.

Chapter 2 - Studies 1-3

Study 1

Relationship between Erythema, Hide Behavior, and Fecal Glucocorticoid Concentrations in the Panamanian Golden Frog (Atelopus zeteki)

Shawna Cikanek[1], Janine Brown[2], Katharine Hope[2], James W Carpenter[1], and Brian Gratwicke[2]

[1] Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS
[2] Smithsonian Conservation Biology Institute, National Zoological Park, Front Royal, VA

ABSTRACT

Sixty Panamanian golden frogs (*Atelopus zeteki*) were transported from the Maryland Zoo, Baltimore, MD, to the Smithsonian Conservation Biology Institute (SCBI), Front Royal, VA. Within 2 weeks of arrival, six frogs died and many others became sick. Gross necropsies of deceased frogs revealed ventral erythema and histopathology confirmed severe dermatitis secondary to water mold, protozoal, and bacterial infections. The remaining frogs were sorted into three health groups depending on the severity of clinical symptoms of skin disease: A--none to mild ($n = 17$); B—moderate ($n = 28$); and C—severe ($n = 9$). We examined whether health status based on dermatitis lesions was correlated with fecal glucocorticoid (GC) concentrations and the amount of time spent in a hide over a 6 week period. There were no correlations between fecal GC metabolites and health status of the animals, between health status and amount of time spent in a hide, or between GCs and the amount of time spent hiding. Thus, neither fecal GC concentrations nor hide behavior were affected by health status due to skin abnormalities.

INTRODUCTION

Stress can be defined as a state in which homeostasis is lost and a stressor is any physical or psychological factor that causes a disruption in homeostasis [14]. The stress response has

evolved as an adaptive mechanism to allow animals to respond quickly to changes in their environment. Thus, stress and stress responses are natural elements of life. Stress hormones cause the mobilization of energy and the temporary suppression of non-essential functions, such as the reproductive and immune systems, so the animal can adaptively respond to a threat [25,27,72,73]. However, chronic stress or repeated exposure to acute stressors can have a negative impact on health and welfare [74,75]. These can include environmental stressors and be harmful for species with limited adaptation capabilities, such as amphibians.

The skin of an amphibian is thin and composed of only a few cells layers, which allows it to modulate a wide array of physiologic functions, including respiration and osmotic regulation [7]. This high permeability, however, also causes amphibians to be more susceptible to sudden environmental changes [7]. Because of this, amphibians are known as the "canaries in the coal mine" of the animal kingdom and are used as biological indicators of overall global health. They are among the first responders to fluctuations in water and air quality, or climate change and habitat obstruction [76] and can be used as an indication of the health of the environment.

In many species, temporary increases in fecal glucocorticoid (GC) concentrations can be used to identify acute stressors [14,38,73,77–79] and provide measurable, noninvasive, insights into conservation and management issues [12,22]. One way to decrease the level of stress in an animal is to provide a place to retreat from stressful stimuli [80–82]. In certain cold-blooded animals, providing a hide, or retreat, can reduce the amount of atypical behavior shown in a captive setting [83]. There is little documentation on the effectiveness of using hides for amphibians, however, the "Guide for the Care and Use of Laboratory Animals" recommends including a place of retreat for amphibians in a captive laboratory setting [84]. One study indicated that providing pipes as refuge for *Xenopus laevis* heightened the physical and social wellbeing of the animals by decreasing the number of aggressive interactions between individuals [84]. According to "Amphibian Medicine and Captive Husbandry," a hide should be incorporated for amphibians to allow an animal to retreat and prevent unnecessary stress [3].

Sixty Panamanian golden frogs (*Atelopus zeteki*) were transported 175 km from the Maryland Zoo, Baltimore, MD, to the Smithsonian Conservation Biology Institute (SCBI), Front Royal, VA. Initial husbandry protocols at the SCBI focused on minimized handling of the frogs and involved an automated misting system. Paper towels and water were changed every 2 weeks and the cages were cleaned and bleached once monthly. The minimal cleaning regimen led to an

overgrowth of microbes and within 2 weeks six of the frogs had died. Gross necropsies revealed all deceased frogs had severe ventral erythema and some had dermal ulcerations. Histopathology confirmed severe dermatitis secondary to water mold, protozoal, and bacterial infections. A full health assessment of the remaining 54 frogs indicated that the majority (~60%) had varying degrees of erythema and dermal ulcerations. Cytological examination of skin sheds were performed opportunistically and confirmed bacterial and fungal dermatitis in some of the remaining frogs. Husbandry protocols were modified to prevent environmental overgrowth of potential pathogens and the affected frogs were treated for bacterial and fungal infections. Once the new protocols were in place, fecal samples were collected daily over a 6-week period for fecal GC analysis.

The objective of this study was to determine whether animal health status based on skin lesion assessment is correlated with fecal GC concentrations and amount of time spent in hide during the study period.

METHODS

Fifty-four *A. zeteki* frogs were housed in a climate controlled room at the SCBI at a temperature between 18°C and 24°C (65°F-75°F). Frogs were maintained individually in mouse cages measuring 29.2 cm x 19 cm x 12.7 cm with low-profile, filter-top lids that were elevated to provide wet and dry areas within the cage. A moist paper towel was provided to maintain humidity in the individual cages [2]. Room and cage humidity were measured continuously using a hygrometer. The diet consisted of crickets (*Achatina domestica*) and/or fruit flies (*Drosophila melanogaster*) fed ad libitum daily. Lighting directly above each rack of frogs was provided by GE Chroma 50 fluorescent tubes[1] on an automated cycle from 0600 – 1800 hr. Tanks were cleaned daily by removing standing water and replacing the damp paper towel. Once a week, frogs were transferred to clean cages that had been disinfected with a 10% sodium hyperchlorite (bleach) solution. Hides were provided in the form of opaque plastic flower pots measuring 5.7 cm wide and 8.3 cm long which were cleaned and disinfected weekly with the cages. Water for the cages was produced by a reverse-osmosis system, reconstituted, and stored

---

[1] General Electric Company, Fairfield, Connecticut.

in a 90 L plastic container with the following chemicals added: 3.56 g calcium chloride, 4.19 g magnesium sulfate, 3.23 g potassium bicarbonate, and 2.69 g sodium bicarbonate.

Each frog was assessed three times each week by a veterinarian and the following criteria were used to classify the frogs into one of three groups based on an erythema health assessment: A--none to mild ($n = 17$); B—moderate ($n = 28$); and C—severe ($n = 9$) (Table 1).

Frogs were treated based on the severity of their disease. Initial treatments included topical applications of silver sulfadiazine ointment, chlorhexidine 0.05% and ciprofloxacin 0.3% (ophthalmic solution applied topically to back) on an as-needed basis. About 60% of the frogs did not respond to initial treatment and were further treated with benzalkonium chloride (1 mg/L bath), itraconazole (0.01% bath), gentamicin (3 mg/mL ophthalmic drops applied topically), and ceftazidime (20 mg/kg intramuscularly) (Table 2).

Fecal samples were collected daily and at the end of the study, eight animals in each group (A, B, or C) that had maintained the same health status throughout the 6-week study period were used for fecal GC analysis. Fecal pellets were stored individually at -20°C and pooled by week to produce enough sample for GC extraction. The extraction method was modified from Brown et al. [28,85] and validated for *A. zeteki*. See Appendix A for detailed fecal GC extraction method and Appendix B for fecal GC validation techniques.

Hide behavior was monitored twice daily for each frog over the 6-week study to determine the relationship between health status and frequency of time spent in hide. Frog position (in or out of the hide) was recorded immediately upon arrival in the frog room at SCBI during morning and afternoon keeper routines to minimize the impact of keeper's presence on behavior results.

The relationship between health status and GC metabolites was calculated using a one-way analysis of variance (ANOVA). Overall hide behavior was analyzed using a one-way ANOVA examining the relationship between health status and time spent in hide per frog per week. Morning versus afternoon hide behavior data was analyzed using a two sample T-test on Minitab software version 14.

RESULTS

A total of 24 frogs remained consistently in one of the three erythema groups for the duration of the 6-week study. The remaining 30 frogs fluctuated between groups and were not

included in the study. Frogs were found in the hides only 2% of the time, which did not vary between morning and afternoon observations ($p = 0.10$). There was no difference between health status and amount of time spent in hide (F (2,23), $p = 0.80$). Furthermore, there were no differences among groups in fecal GC metabolite concentrations ($F(2,138)$, $p = 0.12$), although there was a wide range in mean concentrations (Table 3).

DISCUSSION

This is the first study to investigate changes in adrenal activity in relation to disease in a captive environment, and time spent in hide in an endangered frog species, the Panamanian golden frog. Overall, there was no correlation between the severity of disease and fecal GC metabolite concentrations. Dermatitis in amphibians is a common observation in a captive setting [86–88] with diagnoses including parasitic, fungal, and bacterial overabundance and symptoms ranging from ventral erythema to cutaneous ulcers [89–92]. The animals in this study developed erythema because of water mold, protozoal, and bacterial infections and immediately underwent treatment based on the severity of the symptoms.

Out of the 54 frogs that started the study, 4 improved (went from a higher category to a lower one) during the study while 10 became worse. About half (24) remained in the initial screening group and were used in the data analysis. Based on the lack of a relationship between fecal GCs and the degree to which a frog contracted skin erythema, changes in adrenal function do not appear to be a cause or an effect of this particular skin disease. In other studies, however, a correlation between disease or environmental disruption and GC concentrations could be determined in a variety of species, including amphibians [55,79,93].

 Frogs in "C" groups were handled more frequently than those in the other groups because of the necessity of additional treatments, but this did not affect the GC concentrations in either the short or long-term. One possibility is that frogs in group "C" became accustomed to routine handling. It is well documented that human interaction will cause an elevation in glucocorticoid concentrations in wildlife [30,94,95] and some studies show that an animal is less likely to have an endocrine response to the same stressor after acclimation [12,34]. All frogs in this study were held at least 3 times per week to assess health status for at least two weeks prior to the beginning of the study; thus, by the time the study was initiated, the frogs may have been habituated to human interaction.

The frogs in this study only used the hides 2% of the time so the majority of time was spent outside the hide. A hide is thought to reduce stress by providing the animal a place to retreat when over-stimulated [96]. While many studies have demonstrated a place to retreat decreases the hormonal stress response in mammals [80–82], there is little to no documentation on the effectiveness of hides for amphibian well-being [83]. Two textbooks discuss the reclusiveness of cold-blooded animals and infer an adequate hiding area must be provided [2,3]. A study looking at the behavioral response of the wild eastern fence lizard (*Sceloporus undulates*) found the lizards were more likely to hide when faced with a stressor [97]. However, another study involving captive eastern fence lizards found that providing climbing enrichment similar to that in nature did not affect behavior, health, or the concentration of stress hormones [83]. Our data supported the second study in that no correlation was detected between fecal GCs and hide behavior.

In conclusion, there was no correlation between fecal GC concentrations and hide behavior or erythema severity, or between hide behavior and erythema severity. Data revealed that neither fecal GC concentrations nor hide behavior is affected by health status due to skin abnormalities.

Table 1. Criteria for erythema health assessment in *Atelopus zeteki*.

Characteristic	A (Mild)	B (Moderate)	C (Severe)
Generalized erythema	Very mild flush	Moderate flush	Severe flush
Pigmentation changes	No changes to small areas of white pigment	Moderate amount of white pigmentation on pressure points and around black pigment spots	Large amounts of white pigmentation on pressure points and around black pigment spots
Focal erythema	No focal erythema	Mild to moderate erythema focused around black pigmentation-still appears pink in color	Severe erythema focused around black pigmentation-appears red in color
Skin ulcerations	No skin ulceration	Slight ulceration on feet	Ulcerations on feet and occasionally elsewhere
Increased vascularization (rarely seen)	Little to no vascularization	Slight vascularization	Moderate to severe vascularization

Table 2. **Treatments given and dates administered for varying health statuses in** *Atelopus zeteki.*

Treatment	Dates Treated		
	A (*n* = 8)	B (*n* = 8)	C (*n* = 8)
Ciprofloxacin ophthalmic 0.3% 1 drop TO	5/26/11 - 6/24/11	5/26/11 - 7/6/11	5/21/11 - 7/8/2011
Gentamicin ophthalmic drops 1 drop TO	N/R	7/9/11 - 8/8/11	6/24/11 - 8/8/11
Benzalkonium chloride baths (1-2 mg/L)	N/R	N/R	7/5/11 - 7/11/11
Itraconazole 0.01% baths	N/R	7/26/11 - 8/8/11	7/13/11 - 8/8/11
Ceftazidime 0.2 mg IM 3x weekly	N/R	N/R	7/11/11 - 7/26/11

TO = topically; IM = intramuscularly; N/R = not received.

Table 3. Overall mean (± SE) and mean range in glucocorticoid (GC) concentrations for *Atelopus zeteki* **in each of the erythema health assessment groups.**

Erythema Group	GC (ng/g)	GC (ng/g) Range
A (mild) ($n = 8$)	42.8 ± 3	16.8-197.0
B (moderate) ($n = 8$)	35.0 ± 5	11.4-129.3
C (severe) ($n = 8$)	33.6 ± 8	11.3-102.0

SE=standard error.

Study 2

Evaluating Group Housing Strategies for the Ex Situ Conservation of Harlequin Frogs Using Behavioral and Physiological Indicators

Shawna Cikanek[1], Simon Nockold[2], Angie Estrada[3], Jorge Guerrell[3], Roberto Ibáñez[3], Brian Gratwicke[4], Janine Brown[4], James W Carpenter[1], and Katharine Hope[4]

[1] Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS
[2] Ecology and Environmental Management, York University, UK
[3] Panama Amphibian Rescue and Conservation Project, Smithsonian Tropical Research Institute, Republic of Panama
[4] Smithsonian Conservation Biology Institute, National Zoological Park, Front Royal, VA

ABSTRACT

Harlequin frogs of the genus *Atelopus* are rapidly disappearing from their native habitat in Central and South America due to chytridiomycosis-related declines. We established ex situ colonies of two Panamanian species, *Atelopus certus* and *Atelopus glyphus*, but observed that males fought with each other when grouped together. Housing animals singly eliminated this problem but led to a lack of space to house the collection. To evaluate the potential stress effects of grouping animals, we housed male animals in replicated same-sex groups of one, two, and eight animals and measured behavioral interactions and fecal glucocorticoid (GC) concentrations as a measure of stress. When housed in groups of two or eight, animals initially interacted aggressively, but those instances declined significantly in the first 2 weeks of being housed together. In groups of eight, fecal GCs were significantly elevated during the first week of group housing and were also correlated with the frequency of aggressive interactions observed. We conclude that aggressive interactions in same-sex groups of captive *Atelopus* are an issue that may initially cause stress, but the animals can become habituated within a few weeks and safely be housed in same-sex groups for longer periods of time.

INTRODUCTION

About 45% of all amphibians species have declined in recent years, and over 500 species are regarded by the International Union for Conservation of Nature (IUCN) as critically endangered [48–50]. This has prompted a proactive approach to mitigate the loss of species by creating ex situ assurance colonies of endangered species as part of a global 'Amphibian Ark' effort coordinated through the IUCN [51,54]. *Atelopus* species are a high priority for rescue and assurance populations because of their susceptibility to the invasive fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*), which has devastated naïve upland amphibian communities throughout Panama [52,53]. The Panama Amphibian Rescue and Conservation Project was created in response to *Bd*-related declines and consists of two ex situ facilities in Panama that house populations of amphibians; the El Valle Amphibian Conservation Center (EVACC) in mid-western Panama and the Smithsonian Tropical Research Institute's Gamboa Amphibian Research Center (Gamboa ARC) in Central Panama. Collectively, these facilities house five of the six *Atelopus* species from Panama, *A. zeteki, A. varius, A. limosus, A. certus*, and *A. glyphus*. The sixth known *Atelopus* species, *A. chiriquiensis,* has not been observed since 1996 [52] and may be extinct.

Presently consisting of 30 species from eastern Panama, the ultimate goal of the EVACC and Gamboa ARC is to grow the captive population of each specie to a minimum effective population size of 500 individuals, and maintain those numbers through careful population management [56]. Frogs of this genus typically are housed one per cage [98] because of concerns about territorial aggression [41,47]. However, this limits the number of cages that can be supported at these facilities, and hinders efforts to grow the populations. In general, male frogs prefer to use non-physical displays as a means to avoid direct contact with other males [4,39], but if the density of a population is high then physical confrontation becomes more common [47]. Anurans express their territoriality in a series of steps starting with a sequence of warning calls before engaging in physical combat [45]. *Atelopus* males produce vocalizations including a pulsed or buzz call emitted during male-male vocal interactions that is associated with aggressive encounters as well as whistle calls given prior, during, and after physical combat, and chirp calls produced in crowded conditions in captivity [39,40]. Types of *Atelopus* calls include advertisement, release, territorial, and courtship with the most common being

21

advertisement [39]. In addition to vocalizations, *Atelopus* males use visual signals, such as semaphore foot-raising, to signal antagonistic behavior [40].

One way to overcome space constraints and minimize extended amplexus, or physical embrace, is to house animals in larger, same-sex groups. For example, the Association of Zoos and Aquariums (AZA) golden frog project managed by the Baltimore Zoo has over 2,000 adult *A. zeteki* in over 50 participating zoos and aquaria in the USA, housed in same-sex groups [99]. One question is whether the housing strategy used for the captive US *Atelopus* populations would be directly applicable to the Panamanian species in the Arks because animals reared in captivity and maintained in groups may be better acclimated to such conditions, while wild-caught animals, such as those in the Panama breeding centers, might not. The goal of this study was to determine if *A. certus* and *A. glyphus* could be maintained in same-sex groups without compromising animal welfare, as determined by behavioral observations and monitoring of excreted glucocorticoids (GC) as an indicator of stress.

In many species, temporary increases in GCs can be used to identify acute stressors, while long-term elevations are more likely to indicate the existence of a chronic stressor [14,73]. It is only when stress is prolonged, and the animal is unable to adapt or cope with a perceived stressor, that it becomes distressed [72]. Recent work suggests that measuring GCs can be used to indicate stress, aspects of health status, and response to disease[18]. Glucocorticoid release is the last step of a hormonal cascade that begins in the brain and helps an animal adjust to a stressor [12,72]. An animal's internal response to stress involves the activation of the hypothalamic-pituitary-adrenal axis (HPA), and the release of one of two GC hormones from the adrenal cortex depending on the species; cortisol or corticosterone [23]. These GCs can be measured in samples such as urine, feces, plasma, and blood [24], and there are advantages and disadvantages to each approach. Blood analyses are the most common, but not always the most practical because of the potential stress of sample collection [21]. In small species, such as *Atelopus*, collecting enough blood on a regular basis for hormonal analysis is invasive and unrealistic. A rising awareness of the validity of measuring GC from excreta has led to an increase in noninvasive methods using urine and feces [24]. In this study, urine collection was not possible because the cages contained water which would overly dilute the samples. By contrast, fecal pellets were readily collected, and the technique of using fecal GCs rather than

urine was validated for this study. See Appendix A for fecal GC extraction method and Appendix B for fecal GC validation techniques.

The objective of this study was to determine whether wild-caught *Atelopus* males can be housed together without causing undue stress on the individuals involved as measured by documenting aggressive behavioral interactions and fecal GC metabolite concentrations.

METHODS

Facilities that house wild-caught amphibians from Central America are established in El Valle, Panama and Gamboa, Panama. Permission to establish ex situ colonies of amphibians and house them in groups was approved by the Autoridad Nacional del Ambiente and Animal Care and Use Committee of the Smithsonian National Zoological park (# 09-31).

A total of 44 *A. certus* and 22 *A. glyphus* frogs were used in this study. All frogs were housed individually in small Kritter Keeper[2] containers measuring 28 x 19 x 16.5 cm for at least 1 year before the start of the study. Frogs were collected in the field from the Darien province of Panama. Cages were misted daily and enriched with natural plant leaves (*Philodendron* spp.) and damp brown paper towels as substrate for water uptake and increase humidity in the cage. The tanks were placed on metal racks with fluorescent overhead lighting for 12 hours per day and cleaned twice per week. At the start of the experiment, frogs were removed from the Kritter Keeper[2] containers and placed in numbered glass tanks (size 25 x 53 x 38 cm) with false bottoms and automated misting systems that lightly sprayed the tank interiors for 5 minutes every 2 hours. UV lights supplemented the 12-hour overhead fluorescent lights for eight 45-minute intervals per day. Each tank was furnished with 2 live potted plants (*Philodendron* spp.), rocks, and a water basin. Fecal material was removed manually and tanks were not changed for the duration of the experiment. Frogs were randomly assigned to one of three treatment groups of differing sample sizes, consisting of identical tanks housing one, two, or eight male *Atelopus* frogs*,* respectively, in a completely randomized design with six replicates. Two replicates were filled with *A. glyphus* males and four used *A. certus* males. Black, opaque dividers were placed between tanks to prevent individuals from neighboring tanks from influencing behavior. Frogs

---

[2] Lee's Aquarium and Pet Products, San Marcos, California.

were fed ad libitum with small crickets (*Achatina domestica*) or fruit flies (*Drosophila melanogaster*) dusted with calcium or vitamin supplements four times per week.

A range of territorial and aggressive behaviors were recorded to assess the degree of conflict associated with each group size. Aggressive interactions included fighting, mounting, release call, stalking, and waving (see Table 3). A single observer noted behavior in each tank for 5 minutes twice a day, in the morning between 0700-0830 and in the afternoon between 1400-1530 hr. The order of sampling was randomized to prevent any sequential bias due to time of day. All observations in a single week were summed and divided by the number of frogs in each tank to obtain a total number of aggressive interactions observed per frog per week.

Fecal pellets were collected daily during the 5-week study and stored at -20°C until extraction and analysis of GC metabolite concentrations. Samples were pooled by week to obtain a sufficient weight of fecal material for analysis. Collection began 1 week prior to moving frogs to the glass cages (week 0) to establish baseline GC concentrations. The extraction method was modified from Brown et al. [28,85]. Hormone data are expressed as ng/g dried feces and the mean ± standard error of the mean (SEM). See Appendix A for detailed extraction method.

Behavioral data were analyzed using one-way analysis of covariance (ANCOVA) examining the fixed effects of week, group size, and aggressive interactions. Tank number was incorporated into the model as a random effect. Data collected for aggressive interactions was square root transformed to meet assumptions of homogeneity and normality for analysis of variance (ANOVA) evaluated using least squares. The variance in fecal GC values from smaller group sizes was extremely high due to small total volumes of fecal matter, and was much lower in eight frogs per tank samples. This violated assumptions of homogeneity of variances so we were unable to compare fecal GC levels between group sizes of one, two, and eight animals. Nonetheless, we did test for differences in fecal GCs within each group over time using a one-way ANOVA.

RESULTS

In groups of two and eight *Atelopus* spp., aggressive interactions were initially high during week 1 but then declined over the following weeks (Figure 1). The ANCOVA effects of week on aggressive interactions was significant ($F(1,34) = 32.98$, $p = < 0.01$ as was a reduced

frequency of aggressive interactions in groups of 2 ($F_{(1,10)} = 6.43$, $p = 0.03$), while the correlation between week and group size was not significant ($F_{(1,34)} = 1.09$, $p = 0.30$).

Mean GCs in week 0 (pretreatment) for all frogs was 52.5 ± 4.2 ng/g, whereas the average GC concentration during weeks 1-4 was 46.3 ± 4.7 ng/g. The overall GC average for groups of 8 was 30.2 ± 7.9 ng/g, 55.1 ± 9.3 ng/g for groups of 2, and 41.7 ± 4.3 ng/g for singletons. Average GCs for groups of eight in week 1 was 85.8 ± 12.8 ng/g, and the average GCs for groups of two in week 1 was 60.2 ± 9.1 ng/g (Figure 2). There was no difference in fecal GC metabolite concentrations between frogs housed in groups of two ($F_{(4,22)} = 0.238$, $p = 0.91$) or frogs housed individually ($F_{(4,18)} = 1.00$, $p = 0.44$) over the 4-week observation period. In groups of eight, however, fecal GCs were high during week 1 ($F_{(4,25)} = 5.837$, $p < 0.01$) (Figure 2), but returned to baseline levels by week 2.

The most common behavior observed was physical contact which accounted for 28% of all aggressive interactions included in the ethogram. Interactions observed in groups of 8 during week 1 made up 63% of all antagonistic behavior observed throughout the study whereas groups of two for week 1 only accounted for 15% of the total aggressive interactions observed. All tanks with more than one frog displayed all seven types of aggression at some point during the study. Frequency of aggressive interactions was highest in week 1 and decreased thereafter for frogs in groups of eight, resulting in a positive correlation (r =0.92) between GC metabolite concentrations and aggressive interactions (Figure 5). The correlation between fecal GCs and aggression was not significant for frogs housed in groups of two (r =.098).

DISCUSSION

This study showed that housing male harlequin frogs together in same-sex groups of two or eight animals can lead to aggressive interactions between the frogs, but only for a short period of time. The concurrent increase in fecal GC concentrations observed in groups of eight provides physiological evidence that this group size could be stressful to the animals, but apparently only in the short-term. For groups of two, fewer numbers of aggressive interactions were observed and fecal GC concentrations remained stable. In both groups, the aggressive interactions decreased rapidly over time and frogs appeared to have become acclimated to their new tank mates by week 3, while elevated fecal GC concentrations in frogs in groups of eight were only observed for the first week. Thus, we conclude that after a 2-3-week period of

acclimation, even wild-caught *Atelopus* can be safely housed in groups of up to eight without occurrence of mortality, severe injury, or prolonged sub-lethal stress. This is significant from a conservation perspective because it allows more animals to be housed in a limited amount of ex situ space, increasing the number of animals that can be managed for amphibian conservation and reintroduction efforts.

The only examples of physical aggression were observed in the first 2 days of experimentation. While there is little documentation of hierarchy in amphibians [42], it is possible that the initial increase in aggression was due to expression of dominance by way of territory establishment. When male *Atelopus* meet and vocal interactions commence there are two possible outcomes: the first is that one male will flee while the other pursues without physical confrontation; or the second, a fight will occur [39]. Our data supported the second outcome in that physical confrontation was the most abundant interaction observed. Once the fighting ceased, the territoriality reverted to non-physical displays and eventually no aggressive behavior at all. The initial fighting was reflected in the aggressive interactions observed and fecal GC concentration data in that both were elevated during week 1 of experimentation for groups of two and eight. The number of aggressive interactions and concentrations of GC then declined to baseline and remained low for the duration of the experiment.

There were complications extracting individual fecal pellets from frogs housed individually. Low sample mass has proven to cause artificially high hormone metabolite concentrations in bird feces [100]. We observed comparatively high GC concentrations were correlated with unusually low sample mass, so a fecal pellet cut-off weight was established and any sample below 0.01 g was omitted. A total of seven samples were removed because of low sample mass equaling 0.05% of the data points. Unfortunately, most of these were in the individually housed control tanks leaving only 60% of intended control samples to be analyzed. Because of the high number of control samples removed and variability in the usable samples, we consider the control data set to be unreliable.

The availability of *Atelopus* specimens was also a limiting factor in the experimental design. For future experiments, we recommend one experimental block contain 13 tanks (8 tanks of 1 frog, 4 tanks of 2 frogs, and 1 tank of 8 frogs) instead of the three tanks that were used in this study (1 tank of 1 frog, 1 tank of 2 frogs, 1 tank of 8 frogs). An increased sample size for groups of one and two would have allowed an average cortisol concentration per week to be

calculated. We believe this would have more accurately reflected the effect of housing on GC concentrations and eliminated the variation in data for animals housed individually and in groups of two. By carrying out the ideal experimental design we would have increased the number of specimen involved from 66 to 144. Increasing the number of replicates for this experiment was unrealistic because of space constraints and the limited number of these endangered frogs available.

In summary, this study provides evidence that male *Atelopus* can be housed in larger groups, which will contribute to conservation efforts by expanding the numbers of individuals that can be housed at breeding centers in Panama. Other factors should be considered and monitored when managing any captive collection of amphibians. For example, housing animals in groups may lead to changes in body condition if smaller or non-dominant animals do not compete as well for food. Group housing may lead to increased buildup of gut parasite loads [101] or increased aggressive interactions during the breeding season [46]. Any of these could have an impact on the long-term health of an individual if not carefully managed and monitored by animal care staff.
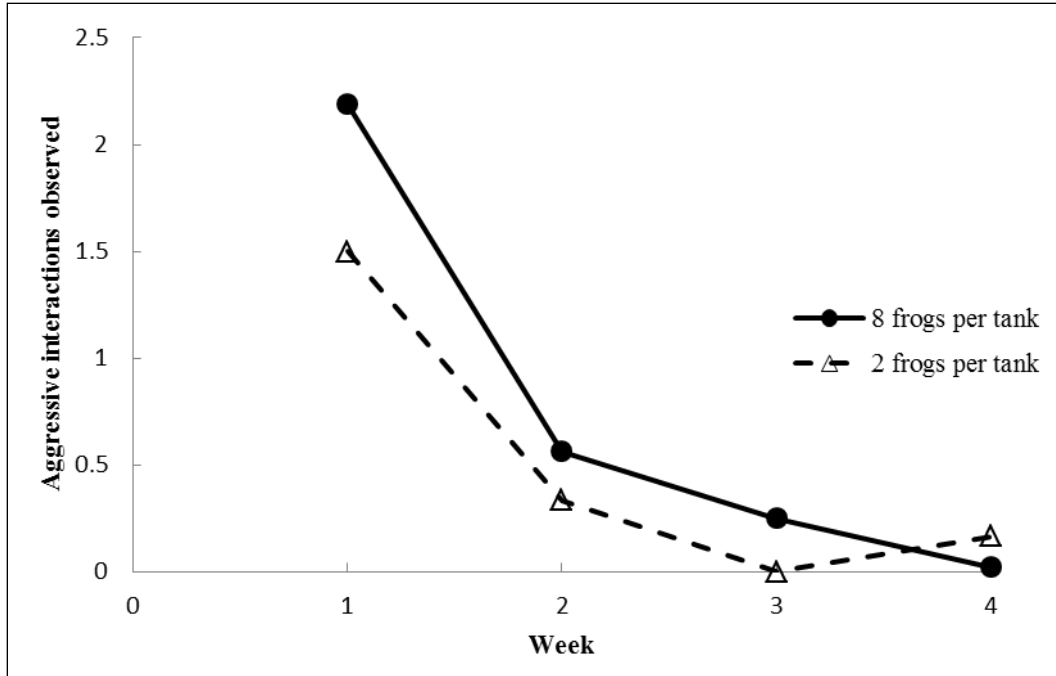
Figure 1. Mean number of aggressive interactions observed per week for *Atelopus certus* and *Atelopus glyphus* housed in groups of two and eight.
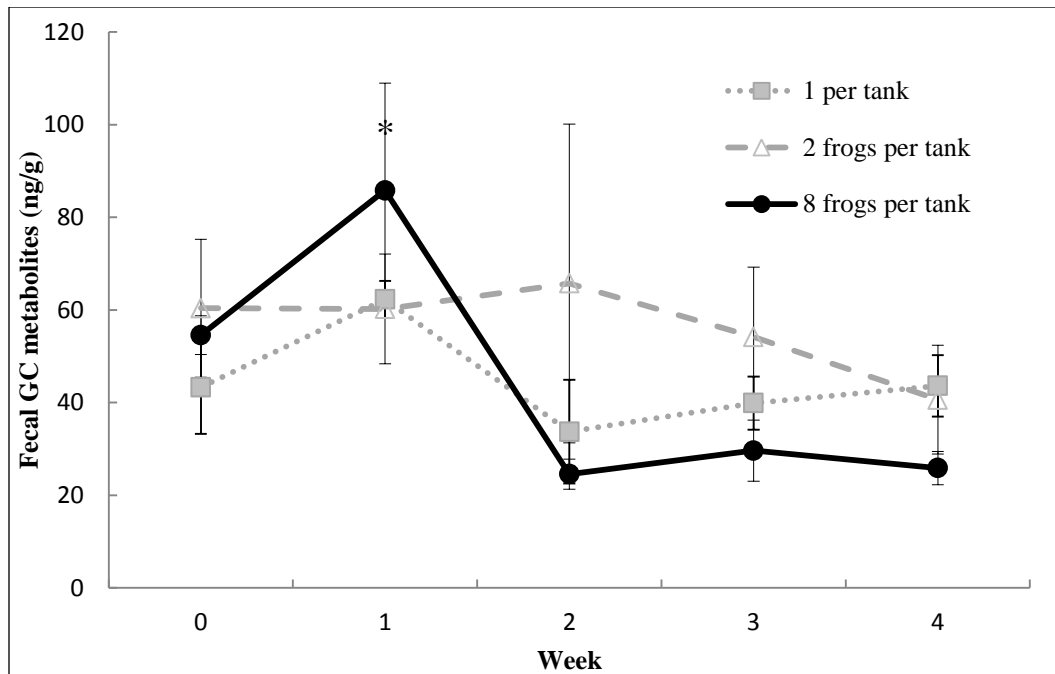
Figure 2. Mean fecal glucocorticoid (GC) concentration per housing group per week in *Atelopus certus* **and** *Atelopus glyphus***. Analysis of variance showing effect of fecal glucocorticoid (ng/g) over time for frogs housed individually** $F_{(4,18)}=1.00$, $p = 0.44$ **in groups two** $F_{(4,22)}=0.238$, $p = 0.91$ **and in groups of eight** $F_{(4,25)}=5.837$, $p < 0.01$.
* Analysis using Tukey's HSD indicates a significant difference ($p < 0.01$).
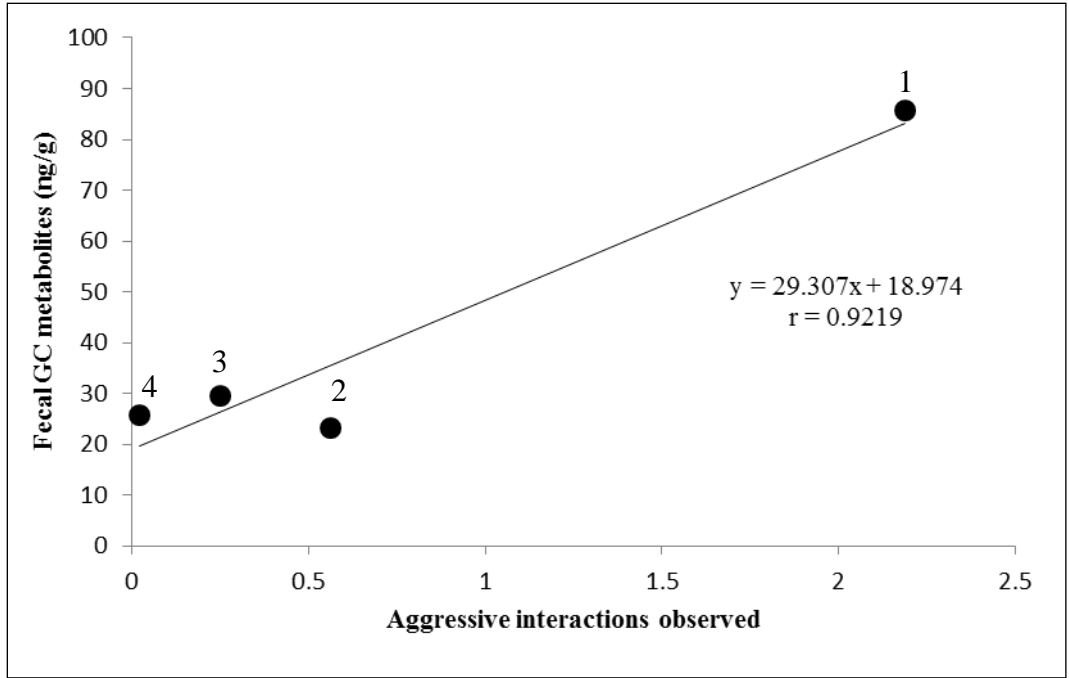
Figure 3. Number of aggressive behaviors correlated with fecal glucocorticoid (GC) metabolite concentrations for *Atelopus certus* and *Atelopus glyphus* housed in groups of eight. Mean number of aggressive interactions observed in the six replicate tanks are plotted against mean fecal cortisol (ng/g). Week number is indicated above each point.

Table 3. Ethogram describing different types of aggressive interactions observed for *Atelopus certus* **and** *Atelopus glyphus*.

Behavior	Description
Fight	Combat involving mouth or front limbs, often flipping of opponent
Mount	>50% of initiators body covers the victim for >5 seconds
Release call	High pitched, weak, peep like call; maximum tally of one per individual
Physical contact	Any remaining forms of physical contact
Stalk	One individual actively follows/chases another for >5 seconds
Wave	Circular movements in front limbs

Study 3

Evaluating Sub-lethal Stress Effects of Antifungal Skin Bacteria Applied to Panamanian Golden Frogs (Atelopus zeteki) as Potential Probiotics to Mitigate the Effects of Chytridiomycosis

Shawna Cikanek[1], Matthew H Becker[2], Brian Gratwicke[3], Janine Brown[4], James W Carpenter[1], and Katharine Hope[4]

[1] Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS
[2] Department of Biological Studies, James Madison University, Harrisburg, VA
[3] Amphibian Rescue and Conservation Project, Smithsonian Tropical Research Institute, Panama
[4] Smithsonian Conservation Biology Institute, National Zoological Park, Front Royal, VA

ABSTRACT

Batrachochytrium dendrabatidis (*Bd*), the causative agent for the disease chytridiomycosis is the only known chytridiomycota to parasitize vertebrates. Because of the unique characteristic of this strain of chytridiomycota to affect amphibians, researchers are trying to identify novel methods to prevent the spread of *Bd*. Multiple strains of naturally occurring antifungal bacteria have been found on wild-caught amphibians and current research is examining ways to augment these natural defenses. The application of anti-*Bd* bacteria on susceptible species could allow individuals to coexist in the wild with *Bd*. Therefore, an experiment was designed based on the hypothesis that an antifungal bacterium from Central America would propagate on *Atelopus* skin. Fifty-six frog species over multiple genera were swabbed in Central America and over 600 bacteria species were isolated. The following four bacteria species; *Pseudomonas* sp., *Pseudomonas putida*, *Chryseobacterium indolgenes*, and *Stenotrophomonas maltophilia* were found to be successful *Bd* inhibitors. This study examined if such treatments impacted animal well-being using fecal glucocorticoid (GC) analyses as stress indicators. There was considerable variation among frogs in the dynamics of fecal GC excretion, but these did not change over time ($p = 0.03$). There also was no significant effect of any one

probiotic treatment ($p = 0.58$) on GC metabolite concentrations. There was no detectable relationship between stress levels and probiotic exposure over time, thus indicating that none of the probiotics had sub-lethal effects on the frogs.

INTRODUCTION

Batrachochytrium dendrabatidis (*Bd*), the causative agent for the disease chytridiomycosis, is the only known chytridiomycota to parasitize vertebrates [1]. Because of the unique characteristic of this strain of chytridiomycota to effect amphibians, researchers are trying to identify novel ways to prevent the spread of *Bd*, a pathogen that is highly virulent and can be found in and around water reservoirs across the globe [58]. *Bd* affects amphibians by keratinizing the epithelial layer of their skin, thus rendering them incapable of gaseous exchange and ultimately leading to congestive heart failure [60]. Upon arrival of the *Bd* fungus in mountainous tropical regions, 50% of the populations and 80% of the individuals in a habitat disappear within 6 months [54]. It was discovered in 2010 that fungal pathogen load has a direct effect on whether a population survives the onset of *Bd* [64]. *Bd* can cause rapid mass extinction or, when fungal zoospore densities on the skin of an amphibian are low, a population can continue to thrive in the presence of the disease for long periods of time [59,63]. It has thus been determined that controlling the amount of zoospores that persist on amphibian skin can be an effective way to stop the spread of *Bd*.

Multiple strains of antifungal bacteria are found on amphibian skin [67] and current research is testing whether these natural defenses can prevent further depletion of the world's amphibians [68,69]. One hypothesis is that reintroducing amphibians with anti-chytrid bacteria will allow individuals to coexist in the wild with *Bd* [71]. One promising bacterial strain was *Janthinobacterium lividum* which was found on the skin of multiple species of North American salamanders and frogs and proved to have anti-*Bd* properties [67,71,102]. Unfortunately, *J. lividum* does not persist on the skin of all amphibians. *Atelopus* frogs from the family Bufonidae are highly susceptible to *Bd* because the neotropical environment they inhabit is conducive to optimal *Bd* growth [53]. Of the 113 known species of *Atelopus*, only 10 have stable populations and their populations are declining at a faster rate than any other family of amphibians [53,69]. In 2012, it was discovered that *J. lividum* does not persist long-term on the skin of the Panamanian golden frog (*A. zeteki*) [55].

This experiment was based on the hypothesis that an antifungal bacterium from Central America would better propagate on *Atelopus* skin. Fifty-six frog species over multiple genera were swabbed in Central America and over 600 bacteria species were isolated. The following four bacteria species; *Pseudomonas* sp., *Pseudomonas putida*, *Chryseobacterium indolgenes*, and *Stenotrophomonas maltophilia* were found to be successful *Bd* inhibitors in vitro. All species were >90% *Bd* inhibitors except *S. maltophilia*. It was included in this study because it was isolated from a wild *Atelopus* species and thus had a good chance of persisting on *Atelopus* skin. The sub-lethal effect of each of these bacteria was assessed by monitoring fecal glucocorticoid (GC) metabolite concentrations to determine impacts of treatment on adrenal activity and stress. A rising awareness of the validity of GC metabolites as indicators of stress in excreta has led to an increase in the use of fecal matter as a determinant of short and sometimes long-term stress [24,37]. This study assessed the ability of each species of bacteria to persist on the skin of *A. zeteki* and analyzed fecal GC concentrations to determine the sub-lethal effects of each bacteria species.

The objectives of this study were: 1) to determine the relationship between fecal GC concentrations as an indicator of stress and probiotic exposure over time; 2) ensure the applied bacteria do not cause any sub-lethal stress to the individuals involved based on fecal GC analysis; and 3) monitor the persistence of each bacterium on *Atelopus* skin.

METHODS

Forty-one adult Panamanian golden frogs were transported from the Maryland Zoo, Baltimore, MD, to the Smithsonian Conservation Biology Institute (SCBI), Front Royal, VA. The 41 frogs were divided into a control group ($n = 9$), or one of four probiotic groups ($n = 8$ each). Frogs were maintained individually in mouse cages measuring 29.2 cm x 19 cm x 12.7 cm with low-profile, filter-top lids that were elevated to provide wet and dry areas within the cage. Frogs were placed on five racks with five containers on one shelf and three (or four for control) on the other. A moist paper towel was provided to maintain humidity in the individual cages [2]. Room and cage humidity was measured continuously using a hygrometer. The diet consisted of crickets (*Achatina domestica*) and/or fruit flies (*Drosophila melanogaster*) fed ad libitum daily. Lighting directly above each rack of frogs was provided by GE Chroma 50

fluorescent tubes[1] on an automated cycle from 0600 – 1800 hr. Tanks were cleaned every day by removing standing water and replacing the damp paper towel. Once a week, frogs were transferred to clean cages that had been disinfected with a 10% sodium hyperchlorite (bleach) solution. Hides were provided in the form of opaque plastic flower pots measuring 5.7 cm x 8.3 cm which were cleaned and disinfected on a weekly basis with the cages. Water for the cages was produced by a reverse-osmosis system, reconstituted, and stored in a 90 L plastic container with the following chemicals added: 3.56 g calcium chloride, 4.19 g magnesium sulfate, 3.23 g potassium bicarbonate, and 2.69 g sodium bicarbonate.

The study was conducted over a 15-week period with no probiotics present the first 3 weeks so baseline cortisol concentrations could be determined. Two weeks prior to probiotic exposure, each frog was rinsed twice in sterile reverse osmosis (RO) water in autoclaved Ziploc[3] containers to remove any extraneous bacteria. Each frog was swabbed 10 times on the belly, 10 times on each thigh, and 5 times on each hind foot to determine normal bacteria load, and then weighed in another autoclaved Ziploc[3] container. Four days prior to inoculation, the bacteria cultures were placed on 1% tryptophan plates. Three days before inoculation, a loop full of culture was placed in 400 µL of 1% tryptone. The cultures were placed on a shaker at 2500 rpm.

The cultures were transported to SCBI on inoculation day after dividing into four tubes containing 50 µL of bacteria, each. The tubes were centrifuged for 10 minutes at 4500 rpm, the supernatant was removed, 10 µL sterile RO water was added to the tube and the centrifugation process was repeated. The final supernatant was removed and the tube was filled with 25 µL sterile RO water. A diluted sample of the culture (1:100) was counted on a hemocytometer (Table 5). An inoculation loop of bacterial solution was added to 1000 mL RO water to obtain 4×10^6 cells/mL. Each frog was weighed and swabbed immediately prior to inoculation and then placed in a Ziploc[3] container with 500 mL sterile RO water and 100 mL of one of the four probiotic solutions. The frogs were placed in the bath for 60 minutes and the water was agitated every 15 minutes to ensure proper coating of the probiotic solution. Finally, the frogs were returned to the cage and placed on the appropriate treatment rack.

Following inoculation, frogs were weighed and swabbed every 2 weeks to monitor the

---

[1] General Electric Company, Fairfield, Connecticut.

[3] S.C. Johnson & Son, Inc., Racine, WI.

bacteria load. Fecal samples were collected daily for 15 weeks from each individual, stored individually at -20°C and then pooled by week to obtain a sufficient weight for GC extraction. The extraction method was modified from Brown et al. [28,85]. See Appendix A for fecal GC extraction method and Appendix B for complete fecal GC validation techniques.

A repeated-measures MANOVA was performed to test the hypothesis that fecal GC concentrations in at least one probiotic treatment group changed significantly over the 15-week experiment. Seven outliers were removed that had GC values over 200 ng/g feces due to a suspected technical error. A Mauchly test was used to test for sphericity to determine whether or not to use a univariate or multivariate approach. Levene's test was selected based on the significant Mauchly test result ($p = 0.01$) indicating that a multivariate test should be used. DNA from the swabs was analyzed with real-time PCR to determine *Bd* infection intensity. An Illumina MiSeq sequencer was used to monitor bacterial community dynamics with barcoded 515F -806R primers.

RESULTS

Fecal GC concentrations averaged 43.7 ± 1.0 ng/g and ranged from 6.2 to 182.8 ng/g. The control group averaged 43.3 ± 2.0 ng/g, *S. maltophilia* averaged 46.5 ± 2.2 ng/g, *Pseudomonas* sp. averaged 42.4 ± 2.0 ng/g, *C. indolgenes* averaged 38.3 ± 2.2 ng/g, and *P. putida* averaged 45.4 ± 3.1 ng/g. Six individuals were considered to have highly variable fecal GCs (SEM > 10.0 ng/g). An example of a frog with variable versus stable GC concentrations can be seen in Figure 4. There was no effect of any one probiotic treatment ($p = 0.33$) on overall GC metabolite concentrations (Figure 5). However, some individual frogs had significantly higher cortisol levels than others ($p < 0.01$) without any discernible trend between individuals. There was a significant effect of time on fecal GC metabolites ($p = 0.03$) and the GCs averaged by week ranged from 26.6 ± 2.3 ng/g to 57.7 ± 5.0 ng/g but not in relation to any specific week ($r = 0.022$). Illumina sequencing revealed that none of the probiotic isolates were found on *A. zeteki* skin at week 4. Probiotic treatments did not affect the microbial community composition on the skin.

DISCUSSION

Fecal GC excretion was dynamic among individual frogs, but there were no consistent changes with respect to probiotic treatment or time. No discernible pattern was detected among frogs with unusually wide GC concentration ranges and these differences are believed to be because of individual frog variability and not in relation to any experimental variables. None of the probiotics used had a significant effect on the frogs involved, possibly because none of the bacteria were found to thrive on *A. zeteki* skin at week 4, indicating a low persistence rate for all bacteria applied in this experiment. There was a significant effect of time on fecal GC metabolites, but not in relation to any specific week, indicating that while there was high variability of GCs from week to week, this was due to individual frog variability and not in relation to bacteria exposure. Individual animals were also found to have a significant effect on fecal GC concentrations yet no pattern could be detected in relation to either bacteria exposure or time.

There was not a detectable relationship between GC concentrations and probiotic exposure over time, thus indicating that none of the probiotics had sub-lethal effects on the frogs. None of the bacteria, however, would be recommended for further studies due to low persistence rates on *A. zeteki* skin. In conclusion, there were no significant findings between time, probiotic or fecal GC metabolites using the probiotic bacteria *Pseudomonas* sp., *Pseudomonas putida*, *Chryseobacterium indolgenes*, and *Stenotrophomonas maltophilia*.
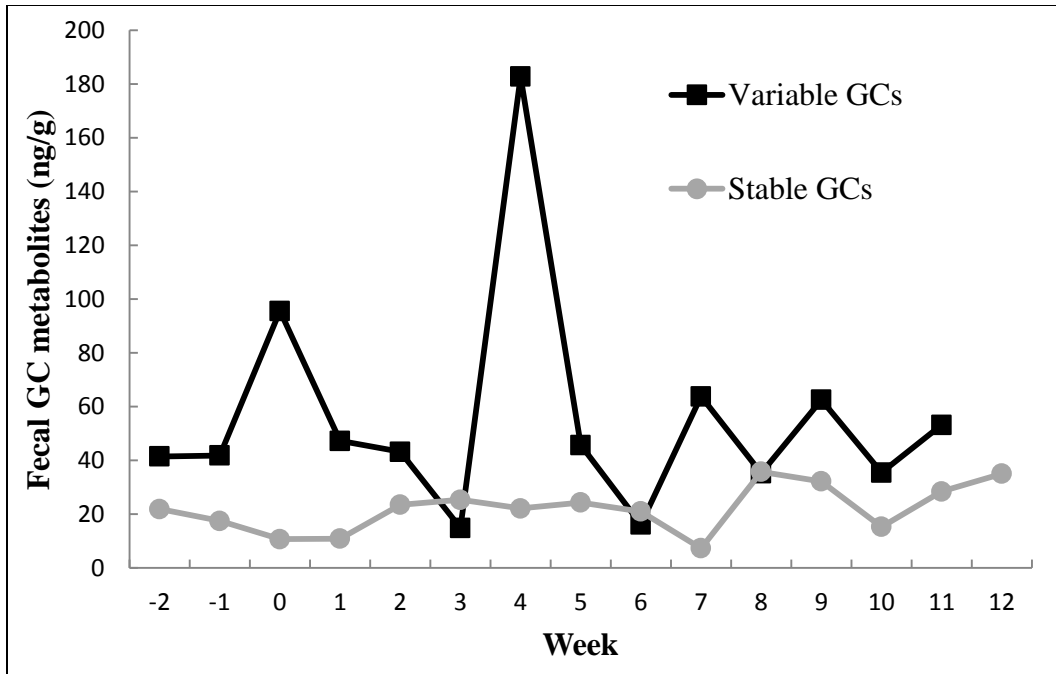
Figure 4. **An example of an individual *Atelopus zeteki* with variable fecal glucocorticoid (GC) metabolites versus an individual with stable GCs.**
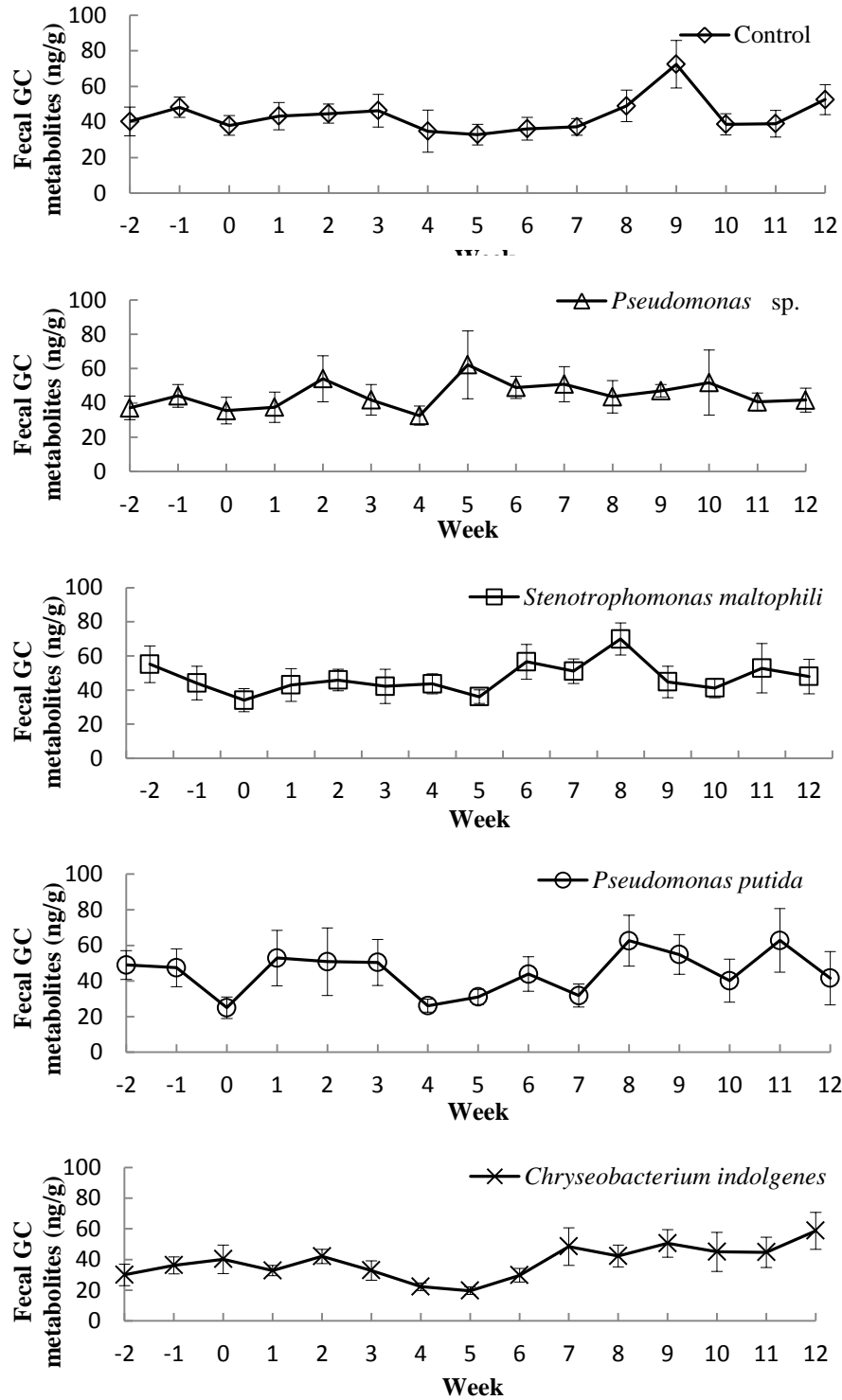
Figure 5. Fecal glucocorticoid (GC) concentrations in *Atelopus zeteki* as a measure of the effectiveness of different probiotic groups against *Batrachochytrium dendrobatidis*. Animals were inoculated with probiotic at week 0.

Table 4. Total count of bacteria and number of cells/mL for each of the four probiotic bacteria used on *Atelopus zeteki* (5 square hemacytometer count and bacteria cells per mL).

Bacteria	Total count (5 squares)	# cells/mL
Stenotrophomonas maltophili	168	8.4×10^8
Pseudomonas sp.	304	1.5×10^9
Chryseobacterium indolgenes	498	2.5×10^9
Pseudomonas putida	311	1.6×10^9

References

1. Chivian E, Berstein A, eds (2008) *Sustaining life: how human health depends on biodiversity*. New York: Oxford University Press. 568 pp.

2. Clayton LA, Gore SR (2007) Amphibian emergency medicine. Vet Clin North Am Exot Anim Pract 10: 587–620 .

3. Wright KN, Whitaker BR (2001) *Amphibian medicine and captive husbandry*. Florida: Krieger Publ Co. 570 pp.

4. Mattison C (2011) *Frogs and Toads of the World*. New Jersey: Princeton University Press. 192 pp.

5. Poll CP (2009) Wound management in amphibians: etiology and treatment of cutaneous lesions. J Exot Pet Med 18: 20–35.

6. Pessier AP (2002) An overview of amphibian skin disease. Semin Avian Exot Pet Med 11: 162–174.

7. Clarke BT (1997) The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biol Rev 72: 365–379.

8. Noble GA, Noble ER (1944) On the histology of frog skin glands. T Am Microsc Soc 63: 254–263.

9. Yotsu-Yamashita M, Kim YH, Dudley SC, Choudhary G, Pfahnl A, et al. (2004) The structure of zetekitoxin AB, a saxitoxin analog from the Panamanian golden frog *Atelopus zeteki*: a potent sodium-channel blocker. Proc Natl Acad Sci USA 101: 4346–4351.

10. Bentley PJ (1998) *Comparative vertebrate endocrinology*. New York: Cambridge University Press. 542 pp.

11. Sheriff MJ, Krebs CJ, Boonstra R (2010) Assessing stress in animal populations: do fecal and plasma glucocorticoids tell the same story? Gen Comp Endocr 166: 614–619.

12. Romero LM (2004) Physiological stress in ecology: lessons from biomedical research. Trends Ecol Evol 19: 249–255.

13. McEwen BS, Wingfield JC (2003) The concept of allostasis in biology and biomedicine. Horm Behav 43: 2–15.

14. Reeder DM, Kramer KM (2005) Stress in free-ranging mammals: integrating physiology, ecology and natural history. J Mam 86: 225–235.

15. Norris DO, Lopez KH, eds (2011) Hormones and reproduction of vertebrates: amphibians. Volume 2. San Diego: Academic Press. 240 pp.

16. Romero LM, Dickens MJ, Cyr NE (2009) The reactive scope model - a new model integrating homeostasis, allostasis, and stress. Horm Behav 55: 375–389.

17. Korte SM, Koolhaas JM, Wingfield JC, McEwen BS (2005) The darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. Neurosci Biobehav 29: 3–38.

18. Goymann W, Wingfield JC (2004) Allostatic load, social status and stress hormones: the costs of social status matter. Anim Behav 67: 591–602.

19. Wingfield JC (2005) The concept of allostasis: coping with a capricious environment. J Mam 86: 248–254.

20. McEwen BS, Wingfield JC (2010) What is in a name? Integrating homeostasis, allostasis and stress. Horm Behav 57: 105–111.

21. Möstl E, Palme R (2002) Hormones as indicators of stress. Domest Anim Endocrin 23: 67–74.

22. Homan RN, Reed JM, Romero LM (2003) Corticosterone concentrations in free-living spotted salamanders (*Ambystoma maculatum*). Gen Comp Endocr 130: 165–171.

23. Cockrem JF, Barrett DP, Candy EJ, Potter MA (2009) Corticosterone responses in birds: individual variation and repeatability in Adelie penguins (*Pygoscelisadeliae*) and other species, and the use of power analysis to determine sample sizes. Gen Comp Endocr 163: 158–168.

24. Sheriff MJ, Dantzer B, Delehanty B, Palme R, Boonstra R (2011) Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166: 869–887.

25. Boonstra R (2005) Equipped for life: the adaptive role of the stress axis in male mammals. J Mam 86: 236–247.

26. De Kloet ER, Vreugdenhil E, Oitzl MS, Joëls M (1998) Brain corticosteroid receptor balance in health and disease. Endocr Rev 19: 269–301.

27. Wingfield JC, Sapolsky RM (2003) Reproduction and resistance to stress: when and how. J Neuroendocrinol 15: 711–724.

28. Graham L, Brown J (1996) Cortisol metabolism in the domestic cat and implications for non-invasive monitoring of adrenocortical function in endangered felids. Zoo Bio 15: 71–82.

29. Keay JM, Singh J, Gaunt MC, Kaur T (2006) Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review. J Zoo Wild Med 37: 234–244.

30. Nilsson PB, Hollmén TE, Atkinson S, Mashburn KL, Tuomi PA, et al. (2008) Effects of ACTH, capture, and short term confinement on glucocorticoid concentrations in harlequin ducks (*Histrionicus histrionicus*). Comp Biochem Physiol 149: 275–283.

31. Zerani M, Gobbetti A (1993) Corticosterone during the annual reproductive cycle and in sexual behavior in the crested newt, *Triturus carnifex*. Horm Behav 27: 29–37.

32. Queyras A, Carosi M (2004) Non-invasive techniques for analysing hormonal indicators of stress. Annali dell'Istituto superiore di sanità 40: 211–221.

33. Narayan EJ, Molinia FC, Christi KS, Morley CG, Cockrem JF (2010) Annual cycles of urinary reproductive steroid concentrations in wild and captive endangered Fijian ground frogs (*Platymantis vitiana*). Gen Comp Endocr 166: 172–179.

34. Bliley JM, Woodley SK (2012) The effects of repeated handling and corticosterone treatment on behavior in an amphibian (Ocoee salamander: *Desmognathus ocoee*). Physiol Behav 105: 1132–1139.

35. Janin A, Léna J-P, Joly P (2011) Beyond occurrence: body condition and stress hormone as integrative indicators of habitat availability and fragmentation in the common toad. Bio Conserv 144: 1008–1016.

36. Coddington EJ, Cree A (1995) Effect of acute captivity stress on plasma concentrations of corticosterone and sex steroids in female whistling frogs, *Litoria ewingi*. Gen Comp Endocr 100: 33–38.

37. Millspaugh JJ, Washburn BE (2004) Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. Gen Comp Endocr 138: 189–199.

38. Wasser SK, Hunt KE, Brown JL, Cooper K, Crockett CM, et al. (2000) A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. Gen Comp Endocr 120: 260–275.

39. Jaslow AP (1979) Vocalization and aggression in *Atelopus chiriquiensis* (Amphibia, Anura, Bufonidae). J Herpetol 13: 141.

40. Lindquist ED, Hetherington TE (1996) Field studies on visual and acoustic signaling in the "Earless" Panamanian golden frog, *Atelopus zeteki*. J Herpetol 30: 347.

41. Cocroft RB, McDiarmid RW, Jaslow AP, Ruiz-Carranza PM (1990) Vocalizations of eight species of *Atelopus* (Anura: Bufonidae) with comments on communication in the genus. Copeia 1990: 631.

42. Duellman WE, Trueb L (1994) *Biology of amphibians*. Baltimore, Maryland: John Hopkins University Press. 670 pp.

43. Mebs D, Ospina SM, Pröhl H, Staudt K (2010) Foraging behaviour and territoriality of the strawberry poison frog (*Oophaga pumilio*) in dependence of the presence of ants. Amphibia-Reptilia 31: 217–227.

44. Carpenter FL, Macmillen RE (1976) Threshold model of feeding territoriality and test with a hawaiian honeycreeper. Science (New York, NY) 194: 639–642.

45. Vilaça TRA, Silva JR dos S, Solé M (2011) Vocalization and territorial behaviour of *Phyllomedusa nordestina* Caramaschi, 2006 (Anura: Hylidae) from southern Bahia, Brazil. J Nat Hist 45: 1823–1834.

46. Crump M (1986) Homing and site fidelity in a neotropical frog, *Atelopus varius* Bufonidae. Copeia 1986: 438–444.

47. Crump ML (1988) Aggression in harlequin frogs: male-male competition and a possible conflict of interest between the sexes. Anim Behav 36: 1064–1077.

48. Baillie JEM, Hilton-Taylor C, Stuart SN (2004) *2004 IUCN red list of threatened species: a global species assessment*. Cambridge, United Kingdom: IUCN. 185 pp.

49. Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, et al. (2004) Status and trends of amphibian declines and extinctions worldwide. Science 306: 1783–1786.

50. Hoffmann M, Hilton-Taylor C, Angulo A, Böhm M, Brooks TM, et al. (2010) The impact of conservation on the status of the world's vertebrates. Science 330: 1503–1509.

51. Gagliardo R, Crump P, Griffith E, Mendelson J, Ross H, et al. (2008) The principles of rapid response for amphibian conservation, using the programmes in Panama as an example. Int Zoo Yearbook 42: 125–135.

52. Lips KR, Diffendorfer J, Mendelson JR, Sears MW (2008) Riding the wave: reconciling the roles of disease and climate change in amphibian declines. PLoS biology 6: e72.

53. La Marca E, Lips KR, Lotters S, Puschendorf R, Ibanez R, et al. (2005) Catastrophic population declines and extinctions in neotropical harlequin frogs (Bufonidae: *Atelopus*). Biotropica 37: 190–201.

54. Mendelson JR, Lips KR, Gagliardo RW, Rabb GB, Collins JP, et al. (2006) Biodiversity. Confronting amphibian declines and extinctions. Science 313: 48.

55. Becker MH, Harris RN, Minbiole KPC, Schwantes CR, Rollins-Smith LA, et al. (2011) Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. EcoHealth 8: 501–506.

56. Frankham R (2002) Predicting extinction risk. Nature 419: 18–19.

57. Longcore J, Pessier A, Nichols D (1999) *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia 91:2.

58. Rollins-Smith LA (2001) Neuroendocrine-immune system interactions in amphibians: implications for understanding global amphibian declines. Immunol Res 23-2: 273–280.

59. Woodhams DC, Vredenburg VT, Simon M-A, Billheimer D, Shakhtour B, et al. (2007) Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. Biol Conserv 138: 390–398.

60. Voyles J, Young S, Berger L, Campbell C, Voyles WF, et al. (2009) Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. Science 326: 582–585.

61. Rachowicz LJ, Hero J-M, Alford RA, Taylor JW, Morgan JAT, et al. (2005) The novel and endemic pathogen hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. Conserv Biol 19: 1441–1448.

62. Alan Pounds J, Bustamante MR, Coloma LA, Consuegra JA, Fogden MPL, et al. (2006) Widespread amphibian extinctions from epidemic disease driven by global warming. Nature 439: 161–167.

63. Vredenburg VT, Knapp R a, Tunstall TS, Briggs CJ (2010) Dynamics of an emerging disease drive large-scale amphibian population extinctions. P Natl Acad Sci USA 107: 9689–9694.

64. Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. P Natl Acad Sci USA107: 9695–9700.

65. Lam BA, Walke JB, Vredenburg VT, Harris RN (2010) Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. Bio Conserv 143: 529–531.

66. Myers JM, Ramsey JP, Blackman AL, Nichols AE, Minbiole KPC, et al. (2012) Synergistic inhibition of the lethal fungal pathogen *Batrachochytrium dendrobatidis*: the combined effect of symbiotic bacterial metabolites and antimicrobial peptides of the frog *Rana muscosa*. J Chem Ecol 38: 958–965.

67. Harris RN, James TY, Lauer A, Simon MA, Patel A (2006) Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. EcoHealth 3: 53–56.

68. Lauer A, Simon MA, Banning JL, Lam BA, Harris RN (2008) Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. ISME J 2: 145–157.

69. Brucker RM, Harris RN, Schwantes CR, Gallaher TN, Flaherty DC, et al. (2008) Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. J Chem Ecol 34: 1422–1429.

70. Becker MH, Harris RN (2010) Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. PloS one 5: e10957.

71. Harris RN, Brucker RM, Walke JB, Becker MH, Schwantes CR, et al. (2009) Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. ISME J 3: 818–824.

72. Sapolsky RM (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21: 55–89.

73. Young KM, Walker SL, Lanthier C, Waddell WT, Monfort SL, et al. (2004) Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. Gen Comp Endocr 137: 148–165.

74. Awerman JL, Romero LM (2010) Chronic psychological stress alters body weight and blood chemistry in European starlings (*Sturnus vulgaris*). Comp Biochem Phys A 156: 136–142.

75. Cyr NE, Romero LM (2009) Identifying hormonal habituation in field studies of stress. Gen Comp Endocr 161: 295–303.

76. Corwin J (2009) *100 heartbeats: the race to save earth's most endangered species*. New York: Rodale Books. 336 pp.

77. Malcolm KD, McShea WJ, Van Deelen TR, Bacon HJ, Liu F, et al. (2013) Analyses of fecal and hair glucocorticoids to evaluate short- and long-term stress and recovery of Asiatic black bears (*Ursus thibetanus*) removed from bile farms in China. Gen Comp Endocr 185: 97–106.

78. Fureix C, Benhajali H, Henry S, Bruchet A, Prunier A, et al. (2013) Plasma cortisol and faecal cortisol metabolites concentrations in stereotypic and non-stereotypic horses: do stereotypic horses cope better with poor environmental conditions? BMC Vet Res 9: 3.

79. Blickley JL, Word KR, Krakauer AH, Phillips JL, Sells SN, et al. (2012) Experimental chronic noise is related to elevated fecal corticosteroid metabolites in lekking male greater sage-grouse (*Centrocercus urophasianus*). PloS one 7: e50462.

80. Kry, C (2007) The effect of hiding enrichment on stress levels and behaviour of domestic cats (*Felis sylvestris catus*) in a shelter setting and the implications for adoption potential. Anim Welfare 16: 375 – 383.

81. Mononen J, Kasanen S, Harri M, Sepponen J, Rekilä T (2001) The effects of elevated platforms and concealment screens on the welfare of blue foxes. Anim Welfare 10: 13.

82. Van de Weerd HA, Van Loo PLP, Van Zutphen LFM, Koolhaas JM, Baumans V (1998) Preferences for nest boxes as environmental enrichment for laboratory mice. Anim Welfare 7: 15.

83. Rosier R (2011) Does environmental enrichment really matter? A case study using the eastern fence lizard, *Sceloporus undulatus*. Appl Anim Behav Sci 131: 71 – 76.

84. Torreilles SL, Green SL (2007) Refuge cover decreases the incidence of bite wounds in laboratory South African clawed frogs (*Xenopus laevis*). J Am Assoc Lab Anim 46: 33–36.

85. Brown J, Wasser SK, Wildt D, Graham L (1994) Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biol Reprod 51: 776–786.

86. Perpiñán D, Trupkiewicz JG, Armbrust AL, Geiser DM, Armstrong S, et al. (2010) Dermatitis in captive Wyoming toads (*Bufo baxteri*) associated with Fusarium spp. J Wildlife Dis 46: 1185–1195.

87. Taylor SK, Williams ES, Pier a C, Mills KW, Bock MD (1999) Mucormycotic dermatitis in captive adult Wyoming toads. J Wildlife Dis 35: 70–74.

88. Cunningham AA, Sainsbury AW, Cooper JE (1996) Diagnosis and treatment of a parasitic dermatitis in a laboratory colony of African clawed frogs (*Xenopus laevis*). Vet J 138: 640–642.

89. Parker JM, Mikaelian I, Hahn N, Diggs HE (2002) Clinical diagnosis and treatment of epidermal chytridiomycosis in African clawed frogs (*Xenopus tropicalis*). Comparative Med 52: 265–268.

90. Fremont-Rahl JJ, Ek C, Williamson HR, Small PLC, Fox JG, et al. (2011) Mycobacterium liflandii outbreak in a research colony of *Xenopus* (*Silurana*) tropicalis frogs. Vet Pathol 48: 856–867.

91. Trott KA, Stacy BA, Lifland BD, Diggs HE, Harland RM, et al. (2004) Characterization of a Mycobacterium ulcerans-like infection in a colony of African tropical clawed frogs (*Xenopus tropicalis*). Comparative Med 54: 309–317.

92. Ford TR, Dillehay DL, Mook DM (2004) Cutaneous acariasis in the African clawed frog (*Xenopus laevis*). Comparative Med 54: 713–717.

93. Wada H, Yates DE, Evers DC, Taylor RJ, Hopkins WA (2010) Tissue mercury concentrations and adrenocortical responses of female big brown bats (*Eptesicus fuscus*) near a contaminated river. Ecotoxicology 19: 1277–1284.

94. Vick MM, Wildt DE, Turner JB, Palme R, Wolfe BA, et al. (2012) Glucocorticoid response to changes in enclosure size and human proximity in the Persian onager (*Equus hemionus onager*). Ann NY Acad Sci 15: 52–61.

95. Champagne CD, Houser DS, Costa DP, Crocker DE (2012) The effects of handling and anesthetic agents on the stress response and carbohydrate metabolism in northern elephant seals. PloS one 7: e38442.

96. Flecknell P (2002) Replacement, reduction and refinement. Gen Comp Bio 19: 73–78.

97. Trompeter WP, Langkilde T (2011) Invader danger: lizards faced with novel predators exhibit an altered behavioral response to stress. Horm Behav 60: 152–158.

98.	Ibanez R (2012) Pers com.

99.	Murphy K (2012) Pers com.

100.	Hayward LS, Booth RK, Wasser SK (2010) Eliminating the artificial effect of sample mass on avian fecal hormone metabolite concentration. Gen Comp Endocr 169: 117–122.

101.	Haislip NA, Hoverman JT, Miller DL, Gray MJ (2012) Natural stressors and disease risk: does the threat of predation increase amphibian susceptibility to ranavirus? Canadian J Zool 90: 893–902.

102.	Becker MH, Harris RN (2010) Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. PloS one 5: e10957.

103.	Suedkamp Wells K, Washburn B, Millspaugh JJ, Ryan M, Hubbard M (2003) Effects of radiotransmitters on fecal glucocorticoid levels in captive dickcissels. Condor 105: 805–810.

104.	Munro C, Lasley BL (1988) Non-radiometric methods for immunoassay of steroid hormones. Non-radiometric assays: technology and application in polypeptide and steroid hormone detection. New York: Allan Liss Corporation. 289–329 pp.

105.	Barry M, Cockrem J, DH B (2010) Seasonal variation in plasma corticosterone concentrations in wild and captive adult Duvaucel's geckos (*Hoplodactylus duvaucelii*) in New Zealand. Australian J Zool 58: 232–242.

106.	Narayan E, Cockrem J (2011) Urinary corticosterone metabolite responses to capture and captivity in the cane toad (*Rhinella marina*). Gen Comp Endocr 173: 371–377.

107.	Narayan E, Moninia F, Cockrem J, Hero J (2012) Individual variation and repeatability in urinary corticosterone metabolite responses to capture in the cane toad (*Rhinella marina*). Gen Comp Endocr 175: 284–289.

Appendix A - Expanded Materials and Methods

Protocol for extracting glucocorticoid (GC) metabolites from feces

For each individual, fecal samples were combined weekly to ensure adequate sample volume [103]. Every solid fecal sample was collected within 12 hours of being voided and all weekly samples were stored frozen in polypropylene tubes until processing. Glucocorticoids were extracted from Panamanian golden frog (*Atelopus zeteki*) feces modified from methods described by Brown, et al. [28,85]. Briefly, wet weekly fecal samples were weighed (mean weight: 0.0360 g, range 0.001 – 0.1333 g) into a 16 x 125 mm borosilicate glass tube and 100 μL ^{3}H-cortisol (~10,000 CPM/100 μL) was added to each tube to monitor efficiency of extraction. Five milliliters of 90% methanol: 10% dH$_2$O (v:v) were added to each sample, tubes were capped, vortexed for 10 seconds then shaken on a large capacity mixer for 30 minutes (Glas-Col, Terre Haute, IN, speed 55, pulse rate 1/second). Tubes were centrifuged at 3500 rpm for 20 minutes, supernatant was recovered, and 5 more mL 90% methanol: 10% dH$_2$O were added to each tube. The pellets were resuspended and the samples were shaken again on a large capacity mixer (30 seconds, speed 55, pulse rate 1/second) and centrifuged for 20 minutes at 3500 rpm. The supernatants were combined, evaporated to dryness under directed air, reconstituted in 1 mL 100% methanol, placed in an ultrasonic cleaner water bath[4] for 10 minutes and dried down. Fecal extracts were reconstituted with 1 mL preservative-free buffer (0.2 M NaH$_2$PO$_4$, 0.2 M Na$_2$HPO$_4$, 0.15 M NaCl; pH 7.0), sonicated for 15 minutes, transferred to polypropylene tubes and stored at -20ºC until analysis. Extraction efficiency was 90 % $\pm$ 0.003 (mean $\pm$ standard error of the mean (SEM)).

Sample extracts were analyzed for GC metabolites following methodology modified from Munro and Lasley [104] using a single antibody cortisol enzyme immunoassay (EIA) employing a polyclonal antiserum (R4866, C. J. Munro, University of California, Davis, CA) and horseradish peroxidase (HRP) ligand (lot 051229, SCBI, Front Royal, VA). The cross-reactivities for R4866 are: cortisol 100.00%, prednisolone 9.90%, prednisone 6.30%, cortisone 5.00%; all other compounds cross-react with the antibody < 1.0% [73]. The standard curve range for the assay is 0.78 – 20.00 ng/mL. Briefly, antiserum was diluted with coating buffer

---

[4] Cole Parmer Instrument Company, Vernon Hills, IL.

(0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6) and adsorbed to NUNC Maxi-sorp flat-bottomed, 96-well microplates overnight at 4ºC. After washing the plate five times (0.05 % Tween 20 in 0.15 M NaCl solution), 50 µL standard, internal control or sample were loaded onto the plate in duplicate, followed by the addition of 50 µL diluted HRP solution to every well. Assays were incubated at room temperature for 1 hour, washed five times and 100 µL of ABTS solution (0.04 M ABTS, 0.5 M H_2O_2 in 0.05 M citric acid buffer) was added to every well. Plates were read on a spectrophotometer (MRX, Dynex Technologies, Chantilly, VA, reading filter 405 nm, reference filter 490 nm) when the optical density (OD) of the 0.00 ng/mL standard reached 1.0 (range: 0.9 – 1.1). Data are reported as ng/g feces. Samples weighing < 0.01g were excluded from the data set because low weight samples consistently exhibited higher glucocorticoid patterns compared to heavier samples [37]. The inter-assay variation on two internal controls (high and low GC concentration) were 7.3 and 8.0 % CV, respectively ($n = 16$). Intra-assay variation between sample duplicates was $< 10\%$ CV.

Appendix B - Validation Procedures

Validation of the use of cortisol in *Atelopus zeteki* feces

Two glucocorticoid assays, a cortisol enzymeimmunoassay (EIA) and a corticosterone radioimmunoassay (RIA), were evaluated for use with Panamanian golden frog (*Atelopus zeteki*) feces. Corticosterone is considered to be the main glucocorticoid (GC) produced in amphibians and assays specific to this hormone are utilized to measure GC concentration in amphibian blood and urine [105,106]. The MP Biomedicals RIA is commonly used to detect corticosterone concentrations in amphibian blood and urine samples, and also glucocorticoid metabolite in feces of several species [38,73]. Narayan et al. [107] determined that a corticosterone EIA was comparable to the MP Biomedicals RIA.

Extraction of fecal GCs was attempted with four different solvent:water ratios. Aliquots of a pooled fecal sample were weighed (0.09 – 0.10 g) and extracted following the procedure described in Appendix A, using one of four solvent:water (v:v) ratios: 90% ethanol:dH$_2$O, 80% ethanol:dH$_2$O, 90% methanol:dH$_2$O and 80% methanol:dH$_2$O. The four subsequent fecal extracts were serially diluted, analyzed on the cortisol EIA, and compared to the standard curve for parallelism (90% ethanol: $r^2 = 0.988$, $F(1,4) = 316.64$, $p < 0.01$; 80% ethanol: $r^2 = 0.980$, $F(1,4) = 397.92$, $p < 0.01$, 90% methanol: $r^2 = 0.995$, $F(1,5) = 1093.27$, $p < 0.01$ and 80% methanol: $r^2 = 0.995$, $F(1,4) = 758.56$, $p < 0.01$). For each extraction method, the linear portion of the slope of the curve was similar to the standard curve (standards: -11.74; 90% ethanol: -11.84; 80% ethanol: -11.78; 90% methanol: -10.98 and 80% methanol: -12.16). Due to comparable parallelisms and slopes among the different solvent:water ratios, the maximum percent binding (%B) of the neat extracts was used to determine that 90% methanol:10% dH$_2$O was the optimal extraction method (90% ethanol: 41.36 %B, 80% ethanol: 38.95 %B, 90% methanol: 31.59 %B and 80% methanol: 37.04 %B). An average recovery of 91% for known concentrations of standard (0.78 – 20 ng/mL) diluted with equal volumes of pooled fecal extract when analyzed on the cortisol EIA indicates low matrix interference.

To compare the MP Biomedicals corticosterone RIA to the cortisol EIA, samples from eleven frogs were analyzed on both assays and the correlation between the two was calculated. The median correlation between the assays for individual fecal GC profiles was high at $r = 0.92$ (range: 0. 57 – 1.00) (Figure 7). Low matrix interference was indicated in the corticosterone

RIA as a result of 88% recovery of known standard concentrations when diluted with equal parts fecal extract pool.

High pressure liquid chromatography[5] (HPLC) was utilized to characterize the numbers and proportions of immunoactive hormone metabolites excreted in *A. zeteki* feces. Three aliquots of pooled fecal samples were extracted as described above, omitting the ^{3}H tracer. The methanol extracts were pooled, dried down under directed air, resuspended in 0.5 mL PBS (0.03 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.15 M NaCl, 0.002 M NaN_3, pH: 5.0), filtered through a C18 Spice cartridge and evaporated to dryness. For chromatographic markers, approximately 3,500 dpm of titrated (^{3}H) cortisol and corticosterone were each added to the extract. The extract was dried down then reconstituted in 0.3 mL methanol (HPLC Grade Methanol, Fisher Scientific, Pittsburgh, PA) and sonicated for 15 min. Then, 0.05 mL of extract was loaded onto a reverse-phase C18 HPLC column (Agilent Technologies, Santa Clara, CA) and a 20-80% linear gradient of HPLC Grade methanol:water over 80 min (1 mL/min. flow rate, 1 mL fractions), which separated the sample extract by polarity. A 0.05 mL portion of each fraction was analyzed for the radioactive hormone markers using a multi-purpose β-radiation scintillation counter (LS 6500, Beckman Coulter, Brea, CA) and the remaining volume was dried down. All fractions were reconstituted with 0.2 mL preservative-free phosphate buffer and analyzed in singlet on the cortisol EIA and corticosterone RIA. Profiles of immunoreactivity and radioactive markers were compared for retention time to characterize fractionated hormone metabolites.

Titrated cortisol eluted at fractions 39 – 41, peaking at fraction 40 while peak radioactive corticosterone eluted at fraction 45 (range: 44 – 46). Immunoactivity on the cortisol EIA indicated the presence of cortisol with a peak at fraction 39. A small amount of immunoactivity at fractions 44 and 45 suggests that the cortisol EIA is able to detect a metabolite that elutes similar to corticosterone. Added immuno activity at fraction 13 indicates an unknown polar metabolite and there were several peaks of uncharacterized non-polar metabolites observed at fractions 54, 59, 66, 75 and 79. Conversely, limited immunoactivity was noted with the corticosterone RIA only at fractions 50, 54 and 59.

Both assays were able to detect similar patterns of hormone excretion (Figure 6), although the cortisol assay appeared to detect higher overall concentrations of metabolites. The

---

[5] Varian ProStar; Varian Analytical Instruments, Lexington, MA.

use of portable and radioactivity-free EIAs advocates for the use of the cortisol EIA over corticosterone RIA in *A. zeteki* fecal GCs, and so it was used for all of the studies in this thesis. However, Narayan et al [107] has indicated the use of corticosterone EIA is comparable to the RIA.
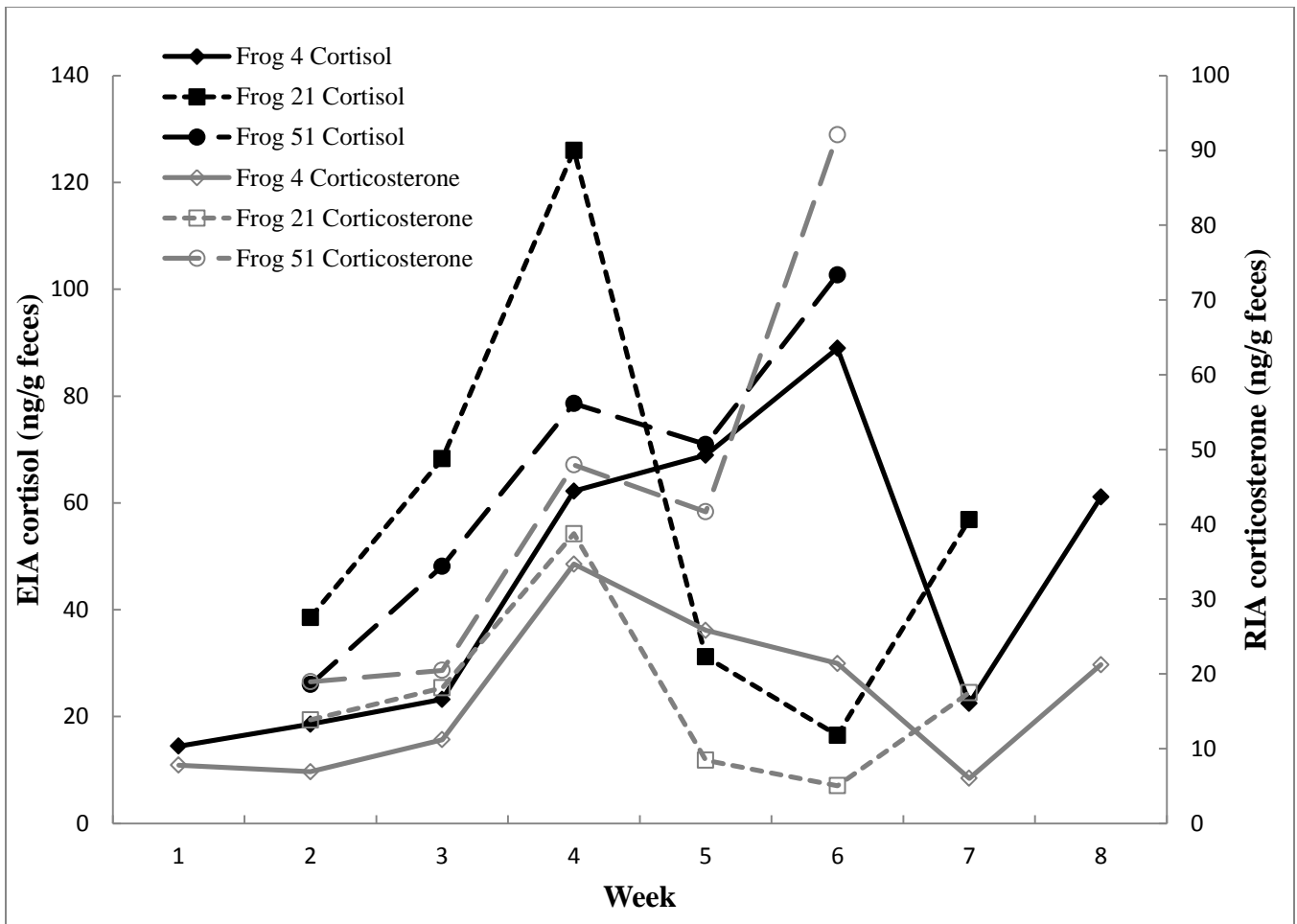
Figure 6. Comparison between fecal cortisol enzymeimmunoassay (EIA) and fecal corticosterone radioimmunoassay (RIA) in individual *Atelopus zeteki*.
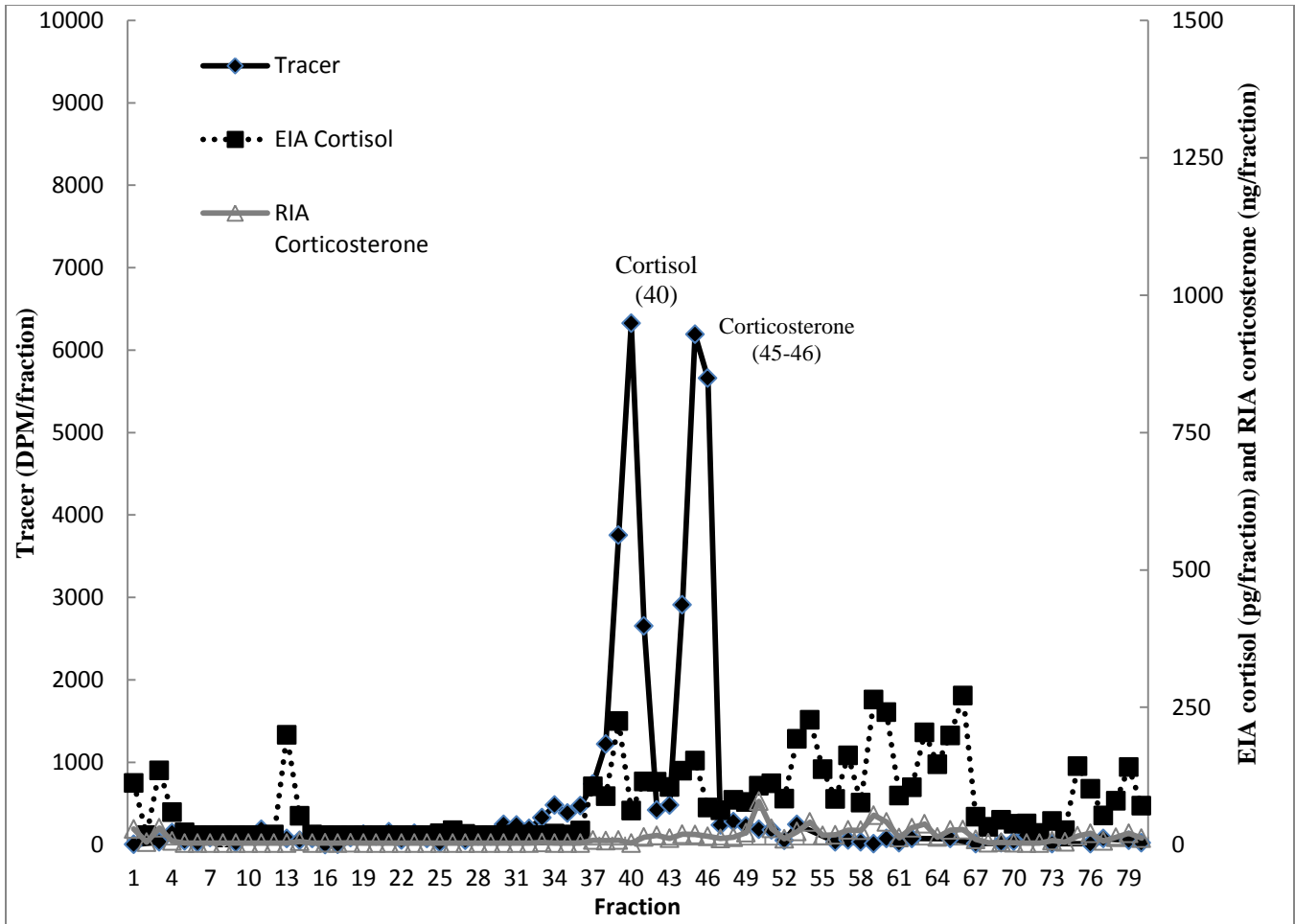
Figure 7. High pressure liquid chromatography (HPLC) results used to determine the numbers and proportions of immunoreactive metabolites in *Atelopus zeteki* fecal extracts. DPM=disintegrations per minute; EIA= enzymeimmunoassay; RIA=radioimmunoassay.