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Anthropogenic and Natural Stressors and Their Effect on Immunity, Reproduction, and the Stress Response

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ANTHROPOGENIC AND NATURAL STRESSORS AND THEIR EFFECT ON

IMMUNITY, REPRODUCTION, AND THE STRESS RESPONSE

by

Lorin A. Neuman-Lee

Dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biology

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ABSTRACT

Anthropogenic and Natural Stressors and Their Effect on Immunity,

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by

Lorin A. Neuman-Lee, Doctor of Philosophy

Utah State University, 2016

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Organisms must be able to cope with many natural and anthropogenic stressors in

order to successfully survive and reproduce. These stressors can come in many forms and

are increasing as anthropogenic activities become more and more prevalent across the

globe. In order to cope with these stressors, organisms must allocate limited energy away

from processes such as reproduction to mount a stress response. This stress response

involves the activation of the hypothalamic-pituitary-adrenal axis and results in a cascade

of hormones and down-stream effects, such as changes in reproduction and immune

function. In order to understand how reptiles and amphibians cope with a variety of

stressors, I conducted seven experiments. I first validated an immune assay which can be

employed across vertebrate taxa and can measure functional immune responses. I then

analyzed effects of natural stressors (wounding, predator attacks, natural toxins, and food

restriction) and/or anthropogenic stressors (restraint and the anthropogenic toxins polybrominated diphenyl ether and indoxacarb) on reptiles and/or amphibians. In measuring many different stressors and several different taxa (the side blotched-lizard, *Uta stansburiana*, the rough-skinned newt, *Taricha granulosa*, the wandering gartersnake, *Thamnophis elegans,* and the common gartersnake, *T. sirtalis*), I hoped to determine if patterns in energy allocation and trade-offs existed on a broad scale. I found that while there are some similarities among the responses, each organism exposed to different stressors had to be examined separately. This supports the emerging consensus that the stress response is extremely context-dependent and responses seen in one context cannot be inferred to other organisms with disparate life-histories, sexes, geographic range, or previous experience. Because of this, researchers must focus on the population in question to assess physiological questions before making management decisions.

(327 pages)

PUBLIC ABSTRACT

Anthropogenic and Natural Stressors and Their Effect on Immunity,

Reproduction, and the Stress Response

Lorin A. Neuman-Lee

Animals must be able to cope with many natural and human-made stressors in order to successfully survive and reproduce. These stressors can come in many forms and are increasing as human activities become more and more prevalent across the globe. In order to cope with these stressors, organisms must allocate limited energy away from processes such as reproduction to mount a stress response. This stress response involves the activation of the hypothalamic-pituitary-adrenal axis and results in a cascade of hormones and down-stream effects, such as changes in reproduction and immune function. In order to understand how reptiles and amphibians cope with a variety of stressors, I conducted seven experiments. I first validated an immune assay which can be employed across vertebrate taxa and can measure functional immune responses. I then analyzed effects of natural stressors (wounding, predator attacks, natural toxins, and food restriction) and/or human-made stressors (restraint and the anthropogenic toxins polybrominated diphenyl ether and indoxacarb) on reptiles and/or amphibians. In measuring many different stressors and several different animals (the side blotched-lizard, *Uta stansburiana*, the rough-skinned newt, *Taricha granulosa*, the wandering gartersnake, *Thamnophis elegans,* and the common gartersnake, *T. sirtalis*), I hoped to determine if patterns in energy allocation and trade-offs existed on a broad scale. I found

that while there are some similarities among the responses, each organism exposed to different stressors had to be examined separately. This supports the emerging consensus that the stress response is extremely context-dependent and responses seen in one context cannot be inferred to other animals with different life-histories, sexes, geographic range, or previous experience. Because of this, researchers must focus on the population in question to assess physiological questions before making management decisions.

To Nick,

who always comes and finds me

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CHAPTER 1

INTRODUCTION

The term "stress" is most generally defined as an occurrence that could potentially shift an organism away from homeostatic balance (Sapolsky et al. 2000; Wingfield 2005). Within this broad definition, there are many types of stress that can cause the organism to react in such a way to compensate for this shift. Both natural and anthropogenic sources of stress exist in nearly every known environment and often interact in complex ways.

A way to determine the balance of these shifts and to measure how well an organism maintains balance is to consider the energetic balance and investment into primary systems that regulate physiological functions, such as digestion, muscle function, reproduction, immunity, and cognition (McEwen & Sapolsky 1995; Romero et al. 2009; Wingfield 2005; Wingfield & Romero 2001). When an organism undergoes a stressful event, the body goes through a cascade of physiological reactions that reallocate energy to vital systems (Sapolsky et al. 2000; Sorrells & Sapolsky 2007). Briefly, the stressor is perceived by the brain and, within seconds, activation of the sympathetic innervation into the adrenal medulla results in the release of catecholamines. Within minutes, the steroid hormone response, the glucocorticoids (GCs), are released into the blood stream via the hypothalamic pituitary adrenal axis (HPA axis; Charmandari et al. 2004). Compared to catecholamines, GCs are better studied due to their persistence in the blood stream (Morilak et al. 2005; Roth et al. 1982) and longer-lasting (i.e., transgenerational) effects (Meaney 2001).

Glucocorticoids include many hormones, although the most noteworthy in the context of stress physiology are cortisol (found in mammals and fish) and corticosterone (found in all other taxa; Moore & Jessop 2003; Wingfield & Romero 2001). These two hormones act in a very similar manner to mediate physiological processes by allocating energy where and when appropriate (McNamara & Buchanan 2005). Their actions vary dramatically depending upon context (such as reproductive status, life history stage and strategy, energetic/nutritional state, previous experience to stress), which can make the interpretation of their levels difficult (Crespi et al. 2013; Crossin et al. 2016; Jaatinen et al. 2013). The most dramatic difference in GC response arises when comparing chronic or acute exposure to stress (Skórzewska et al. 2006), although differences in the response can be detected depending upon sex (Kajantie & Phillips 2006; Kudielka & Kirschbaum 2005), life-history stage (Crespi et al. 2013), life-history strategy (Monaghan 2014; Schwartz & Bronikowski 2013), previous exposure (Figueiredo et al. 2003; Rich & Romero 2005), intensity of stressor (McCormick et al. 2015), and/or reproductive status (Berger et al. 2005; Bokony et al. 2009).

Stress Response, Reproduction, and Immunity

The classic view of natural stress to an organism involves other co-occurring physiological processes, especially reproduction and immune function (Carlton et al. 2014; French et al. 2007; McNamara & Buchanan 2005; Moore & Jessop 2003; Zera & Harshman 2001). While other physiological processes can cause the body to deviate from homeostasis, these two processes have been the best studied and require a significant

investment of energy (Bonneaud et al. 2003; Gittleman & Thompson 1988). In addition, both reproduction and immunity are not always essential for survival (compared to physiological processes such as digestion and cognitive function; Wingfield & Romero 2001). However, an organism must invest at least some energy into immunity in order to combat foreign pathogens sufficiently to survive and reproduction if it will pass on any genes. Therefore, these two "optional" systems are often competing for limited resources and provide an excellent way to measure the level of stress, or extra energetic requirement, for an organism (Carlton et al. 2014).

The effect of GCs on reproduction is complex given that some elevation of GCs seems to be required for optimal reproductive output, but too much elevation of GC can cause a decrease or complete cessation of reproductive effort (Wingfield & Sapolsky 2003). This relationship is likely due to the necessary mobilization of energy to reproductive systems by GCs, but if too much energy is required to cope with other stressors, then reproductive effort is reduced (Wingfield & Sapolsky 2003).

A further complication to this relationship is the fact that life history can dictate how much energy should go into reproduction during any given time period. For organisms that have short life spans, the major priority is to reproduce quickly and thus they may display a different pattern of GC/reproductive output relative to an organism with a long life span allowing many opportunities to reproduce (Palacios et al. 2012).

This relationship between energetic spending and life span also is important for understanding the trade-offs between immune function and reproduction during a stressful event (GC elevation). For organisms with shorter life spans, less energy is

typically required for immune function (self-maintenance) because the organism will not live for many reproductive seasons. However, many longer-lived organisms must invest more into the immune system to ensure many prolific reproductive seasons (Lucas & French 2012; Palacios et al. 2012). This trade-off is often considered to be mediated by GCs, although empirical data is still lacking.

In the absence of reproduction, GCs and immune function are still inextricably linked. This relationship is considered highly context-dependent, most strongly with duration of GC exposure. During acute stress, immune function is often augmented, while it is depressed during chronic GC exposure (Dhabhar 2002; Dhabhar 2009; Dhabhar & McEwen 1997).

Sources of Natural Stress

While reproduction and immune function are the best-studied physiological processes in relation to stress, many other natural factors can elicit a stress response. For example, food limitation is known to be stressful (Clinchy et al. 2013). Food limitation is a singular factor in that the lack of food is, itself, stressful, but also energetically limiting, thus altering the stress response dynamic (Clinchy et al. 2004). Given that the primary action of GCs is to mobilize energy, having a reduced pool of resources can change the level of response that the organism can achieve (McNamara & Buchanan 2005).

Predation is a ubiquitous challenge that most organisms face. Organisms have a variety of behavioral strategies to escape predation, from fleeing, hiding, and/or performing displays. All of these strategies could be related to the stress response and

production of GCs (Wingfield 2005). Although not well-studied, there is evidence that

different antipredator strategies elicit different stress responses (Øverli et al. 2004; Øverli

et al. 2007; Thaker et al. 2009).

Closely related to antipredator behaviors, some organisms produce noxious and/or

toxic compounds that deter potential predators (Brodie 1968). The stress response is

likely not involved when organisms have these compounds available regardless of the

threat. Little is known about the mechanisms that control this release, but, given the

stressful nature of a predatory attack, GCs may be involved.

Conversely, many organisms have evolved resistance to these antipredator toxins

(Brodie & Brodie 1990; Hutchinson et al. 2007). Therefore, one could expect that this

resistance confers an added benefit of an organism not perceiving the toxin as a threat—

and thus not needing to elicit a stress response. Theoretically, organisms with different

levels of resistance to a certain toxin would have varying stress responses to a similar

exposure. This ability to modulate the stress response would allow organisms to not

overreact or underreact to a stressor (Charmandari et al. 2004; Romero et al. 2009).

Sources of Anthropogenic Stress

Anthropogenic stressors enter our environment at an ever-increasing pace and

their effect on the physiology of organisms remains largely unknown (Baker et al. 2013).

Virtually every ecosystem is impacted by a variety of anthropogenic factors, including

chemicals, habitat destruction, and climate change. Additionally, there are unknown

interactions between and within anthropogenic and natural stressors that are nearly

impossible to determine, much less quantify. The first steps in understanding how these anthropogenic stressors influence the physiology of an organism is to examine stressors individually and then combine stressors to examine interactive effects (Angelier & Wingfield 2013).

Contaminants

A major anthropogenic stressor is that of a high level of synthetic chemical contamination entering our environment (Cooke et al. 2004; Wikelski & Cooke 2006). From industrial waste to pharmaceuticals to agricultural runoff, contaminants are entering our environment at an astonishing pace (Kolpin et al. 2002). Unfortunately, little is known about the functional effects of most of these chemicals, and the regulatory processes are riddled with potential problems, such that some chemicals may not be tested in a manner that protects humans or the environment in the most feasible manner (Boone et al. 2012).

Minimal research has been conducted on the effects of anthropogenic compounds on the HPA axis and downstream systems (Cooke et al. 2004). However, literally thousands of chemicals enter the environment and have very different mechanisms of action, which make generalizations extremely difficult, if not impossible (Crisp et al. 1998).

Anthropogenic chemicals that have similar mechanisms of action to natural chemicals have been the subject of intense research in entomology and pest control. Due to structural or mechanism of action similarities, insects that have developed resistance to

natural toxins (typically plant defenses) may have resistance to anthropogenic chemicals (reviewed in Després et al. 2007). There are several well-documented examples of vertebrates which have evolved resistance to natural compounds, such as tetrodotoxin (TTX; Brodie et al. 2002; Feldman et al. 2009)) and bufadienolides (Hutchinson et al. 2007; Mori et al. 2012). However, the relationship between the resistance to these natural chemicals (which may elicit less of a stress response in resistant animals) and similar anthropogenic chemicals has not yet been examined. Theoretically, if the stress response is reduced or absent in resistant animals, anthropogenic chemicals of similar structure or mechanism of action may also show a reduced or absent stress response, as seen in insects.

Habitat destruction

Habitat destruction is a major contributor to wildlife decline, and several studies have shown that various forms of habitat destruction elicit a greater stress response in vertebrates (reviewed in Wikelski & Cooke 2006). An altered stress response has been associated with habitat destruction ranging from physical displacement through urbanization (French et al. 2008), invasive organisms (Trompeter & Langkilde 2011), increase in pathogen spread (Gabor et al. 2013), excessive tourism (French et al. 2010), and destruction of habitat (i.e., deforestation or wetland drainage; Wikelski & Cooke 2006).

Climate change

The final major anthropogenic stressor facing wildlife today is that of global climate change (Sutherland et al. 2014). Changing temperatures and precipitation patterns have already begun to affect many sensitive species and the rate and amount of alterations to species is likely to rise (Walther et al. 2002). Many of these changes are somewhat subtle compared to other measured anthropogenic stressors and can therefore be difficult to track and measure. Long-term monitoring of populations may allow scientists to understand what the effects of climate change are on the stress physiology and energetic requirements in various species (Meylan et al. 2012).

Reptiles and Amphibians

Reptiles and amphibians ("herps") are one of the least studied vertebrate groups in terms of stress physiology. Reptiles and amphibians typically receive less attention than mammals, birds, and fishes due to a general lack of charismatic species and economic potential (e.g. Hopkins 2000; Weir et al. 2015; Zimmerman et al. 2010). However, given their location in the phylogenetic tree, they can make excellent species for studying physiology (Moore & Jessop 2003). For example, their endocrine physiology is, for the most part, very similar to birds (Moore & Jessop 2003). Immune physiology is an area of potential exploration as little is still understood about reptilian immunology (Zimmerman et al. 2010).

For studying the stress response, both reptiles and amphibians are excellent model organisms for a wide variety of reasons. Many common species are easy to collect and sample (Brasfield et al. 2004; Hopkins 2000). Additionally, there are many highly

threatened and endangered species that make these same common species valuable for
conservation work (Gibbons et al. 2000; Sutherland et al. 2014). Many herps have
territories or return annually to hibernaculum or breeding ponds, making sampling the
same individuals over time possible (Russell et al. 2005). Especially with respect to
endocrine and innate immune physiology, it is feasible to use already established
protocols for measuring hormones and innate immunity (French et al. 2010; French &
Neuman-Lee 2012; Neuman-Lee et al. 2015; Zimmerman et al. 2010).

Drawbacks to using herps in physiological research still exist. For example, little
is known about basic physiology, so experiments exploring energetic levels (Nagy et al.
1999), osmoregulatory properties (Shoemaker & Nagy 1977), hormone levels (Crews et
al. 1984; Whittier et al. 1987), and reproduction (Moore & Miller 1984; Moore et al.
2000) must often be done prior to more extensive and more detailed questions can be
asked. Further, while many protocols and assays do work for herps, there are many that
are difficult (or impossible) to apply to herp tissue (Zimmerman et al. 2010).

In my dissertation research, I applied a wide variety of stressors and apply them to
several species of reptile and amphibian. Because these studies have many different
components, it is possible to begin to draw more general conclusions as to how stress
physiology and downstream effects impact the physiology and life history of reptiles and
amphibians. To this end, I present seven studies which provide a clearer picture of how
these organisms react to disparate stressors.

Chapter 2— Novel ex vivo method for microbiocidal activity across vertebrate species

In this chapter, I describe the validation of a microplate innate immune assay which I used in most of my research. This assay, which was based on a previous protocol (Liebl et al. 2009), has enabled me to measure bactericidal ability in plasma samples from many species. This is a functional assay, meaning that many parts of the innate immune system work to combat bacteria, much as they would *in vivo*.

Chapter 3— Wound healing reduces stress-induced immune changes: Evidence for immune trade-offs in the side-blotched lizard

This study measured innate immune ability when an organism is challenged with an acute stressor while experiencing an ecologically relevant immune stress—wounding. Wounds are common in wildlife, especially in species that engage in combat over territories and/or mates. We applied acute stress (thus raising the glucocorticoid corticosterone (CORT) levels) after animals were wounded or given a sham-wound. We then measured the rate of healing and bactericidal ability. This study sought to determine if 1) wounding in itself changes the stress response and 2) how other immune functions were changed after wounding. We found that individuals that had a higher stress response (as measured by an increase of CORT after stressing from baseline levels) had faster healing rates, indicating an increased mobilization of energy to healing the wound. While we found that bactericidal ability was initially decreased in wounded animals after the acute-stress challenge, overall bactericidal ability was positively correlated with the rate of healing during the initial immune stages. These findings taken together indicate that

CORT is a potential mediator for immune function and that our measure of innate immunity is a functional measure of innate immune components.

Chapter 4— Effects of antipredator behavior and tetrodotoxin concentrations on stress response in Rough-skinned newts

Rough-skinned newts (*Taricha granulosa*) are famously known for producing high amounts of the toxin tetrodotoxin (TTX). Much research has been conducted on the evolutionary origins and benefits of TTX production, but as of yet no one has examined potential physiological costs of TTX production. In tandem with TTX production, TTX secretion is coupled with an elaborate antipredator display, known as the unken reflex (Johnson & Brodie 1975). While predatory stimuli clearly initiate this behavior, nothing is known about the physiological mechanisms behind the stimulation. This study sought to examine if circulating CORT was related to 1) the amount of TTX produced by an individual newt and 2) the behavioral display of the unken reflex. We conducted two experiments. The first examined the amount of TTX produced in relation to circulating CORT levels after a predatory stimulus. The second sought to measure potential upstream factors in the behavioral modulation by injecting newts with CORT, adrenocorticotropic hormone (ACTH—the upstream hormone of CORT), a CORT-blocker metapyrone, or a control. We found that endogenous CORT levels were not related to TTX production or duration of unken reflex. However, exogenous CORT and ACTH caused an inhibition of unken reflex. This study provides evidence that CORT plays a role in antipredator behavior in these newts.

Chapter 5— To stress or not to stress: Physiological responses to tetrodotoxin in resistant gartersnakes vary by sex

Several species of gartersnakes are in the midst of a co-evolutionary arms race with the Rough-skinned newt. As gartersnakes develop resistance to the levels of TTX in their prey (newts), the newts have developed more concentrated levels of TTX (Brodie & Brodie 1990). This arms race has resulted in populations of highly toxic newts and highly resistant snakes (Brodie et al. 2002). As with newts and TTX production, the evolutionary implications have been studied, while the physiological costs remain undetermined. In this study, we sought to determine if exposure to TTX elicited a stress-response and if the level of resistance, which is variable in the population, was related to the response. We also measured bactericidal ability to determine if this downstream physiological process was influenced. We found that males and juveniles had no significant stress response to increasing doses of TTX. However, we found that females had a strong CORT response to all levels of TTX. This may be due to the fact that CORT and the stress response is highly context-dependent and female and male snakes have very different life-history strategies and traits.

Chapter 6— Comparing the natural and anthropogenic sodium channel blockers tetrodoxoxin and indoxacarb in gartersnakes

As more anthropogenic chemicals enter our environment, we occasionally may have the opportunity to compare synthetic chemicals to existing chemicals to which organisms have already been exposed and adapted. The relatively new pesticide,

indoxacarb (INDOX) acts by binding to sodium channels in insects, specifically in lepidopteran larvae. The natural toxin, tetrodotoxin (TTX), is found throughout the terrestrial, aquatic, and marine environment primarily as a defensive toxin. TTX also works to bind sodium channels. Gartersnakes in the Pacific Northwest of the United States of America have adapted a resistance to TTX due to their predatory relationship with Rough-skinned Newts, which are chemically defended by TTX. Therefore, gartersnakes allow us to look at the physiological effects of both a novel chemical (INDOX) and a chemical to which they have adapted (TTX). We examined individual snake responses to both TTX and INDOX using the physiological parameters of corticosterone, testosterone, and innate immunity. We found that TTX did not elicit a deviation from baseline measurements for any of the three parameters, but INDOX significantly increased corticosterone and innate immunity. This may indicate that chemicals with similar mechanisms of action do not always have the same impact on an organism.

Chapter 7— Physiological effects of polybrominated diphenyl ether (PBDE-47) on pregnant gartersnakes and resulting offspring

Anthropogenic chemicals provide a unique environmental challenge to organisms. These chemicals may alter the physiology due to their mechanism of action, but also due to their influence on other physiological processes, such as the stress response. In this study, we examined the chronic effects of the flame retardant polybrominated diphenyl ether (PBDE-47) on pregnant gartersnakes. We dosed these females from the beginning

of their pregnancy to parturition and measured their stress response, thyroid follicular

height, bactericidal ability, reproductive output, and propensity to resorb their follicles.

We also measured the resulting offspring (level of CORT, bactericidal ability, sex and

size differences). We found that there might be subtle effects on the mother in terms of

increased body mass and higher thyroid follicular height, although our other measures

were not distinguishably different from control. Offspring born to the BDE-47 treated

females had an increased size. These results show that snakes are extremely resilient, but

also that subtle effects may occur when exposed to ecologically relevant levels of

anthropogenic toxins.

Chapter 8— Interactions between food restriction and chronic stress influence investment in immune function in gartersnakes

In this final study, we examined the influence of chronic restraint stress and food

restriction on energy mobilization, the stress response, and immune function in

gartersnakes. Snakes are infrequent eaters compared to most vertebrates and thus provide

an interesting model for examining food restriction. Snakes were assigned to one of four

groups (no stress/food, no stress/no food, chronic stress/food, chronic stress/no food) and

multiple physiological functions were measured after six weeks. The efficacy of the

stress response in relation to these many variables was measured by taking a blood

sample before and after a 30-minute restraint stressor. This study showed that food

restriction and chronic stress do influence innate immune function, stress reactivity, and

energetic metabolites (triglycerides and glycerol). These results show that the stress

response is heavily context-dependent and relies heavily on resource availability and duration of current stressors.

Chapter 9— Conclusion

In my concluding chapter, I review the studies that I have conducted over the past five years and how their findings shed light on the interconnectedness of stress physiology with other key physiological systems and behavior. Further, I explore the future avenues to where this basic research can lead.

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CHAPTER 2

IMPROVED EX VIVO METHOD FOR MICROBIOCIDAL ACTIVITY ACROSS

VERTEBRATE SPECIES[1]

The field of ecoimmunology is currently undergoing rapid expansion, whereby biologists from a wide range of ecological disciplines are increasingly interested in assessing immunocompetence in their study organisms. One of the key challenges to researchers is determining what eco-immune measures to use in a given experiment. Moreover, there are limitations depending on study species, requirements for specific antibodies, and relevance of the methodology to the study organism. Here we introduce a novel ex vivo method for microbiocidal activity across vertebrate species. The utility of this assay is that it determines the ability of an organism to remove a pathogen that could be encountered in the wild, lending ecological relevance to the technique. The applications of this microbiocidal assay are broad, as it is readily adaptable to different types of microbes as well as a wide variety of study species. We describe a method of microbiocidal analysis for use on a microplate reader that will enable researchers across disciplines to effectively employ this method to accurately quantify microbial killing ability, using readily available microplate absorbance readers.

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Novel ex vivo method for microbiocidal activity across vertebrate species.

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INTRODUCTION

It is becoming increasingly apparent that immune responses play an important role in an organism's physiological, biochemical, and behavioral responses to its environment, and thus, have the potential to shape the evolution of life history strategies (Boughton et al., 2011). "Immunocompetence," an individual's capacity to mount an appropriate immune response following exposure to a pathogen, is a critical aspect of disease resistance and, thus, survival (Graham et al., 2011). Therefore, biologists from a wide range of ecological disciplines are increasingly interested in assessing immunocompetence in their study organisms. However, one of the major challenges to researchers is determining what measures to use in a given experiment (Demas et al., 2011). Further there are limitations depending on study species, requirements for specific antibodies, and relevance of the methodology to the study organism.

The microbiocidal assay historically referred to as the bacterial killing assay, measures the capacity to fresh whole blood or plasma to kill microbes *ex vivo* (Millet et al., 2007; Tieleman et al., 2005). However, the utility of this method goes beyond measuring bacterial killing to many different types of microbes and we will therefore refer to it heretofore as the microbiocidal assay. One of the primary benefits of using the microbiocidal assay instead of other measures of immune function is that it determines the ability of an organism to remove a pathogen that could be encountered in the wild. This approach provides an environmentally-relevant immune response. Additionally,

several immune components are measured in this immune challenge. Phagocytes (e.g., macrophages, heterophils, and throbocytes), opsonizing proteins (complement and acute phase proteins), and natural antibodies (predominantly immunoglobulins M and A, IgM and IgA) can be assessed, depending on the type of microbe and whether whole blood or plasma is used. Consequently, a major advantage to this method of immune function assessment is that a variety of different microbes can be used to test functional responses of different specific immune components. For example, unlike many other immune measures, such as total hemolytic complement activity, the killing of the bacteria *Escherichia coli* also relies on the presence of natural antibodies and phagocytes, providing a more integrative measure of immunity while also providing an indication of complement activity. These benefits are in contrast to many other assays that only assess isolated immune components (e.g., lymphocyte proliferation) or responses to relatively artificial antigens and/or mitogens, (e.g., phytoheamagglutinin).

A further advantage to this method is that no specific antibodies are required for this procedure. Therefore, the microbiocidal assay is very adaptable, not species specific, and can be used in a number of species. For example, in the current paper we have validated this assay on non-traditional amphibian (rough skinned newts, *Taricha granulosa*), reptilian (garter snakes, *Thamnophis elegans*), avian (house finches, *Carpodacus mexicanus*), and mammalian (coyotes, *Canis latrans*) species. The selection of this wide range of taxa, with different life histories, from a variety of environments, and with varying blood volumes, helps to demonstrate the applicability of the microbiocidal assay across a range of different taxa.

Additional advantages to the microbiocidal assay are its simplicity, short duration, small sample volume requirements, and that it requires only a minimal amount of specialized equipment to perform. Ideally, a sterile laminar flow hood is used; however, a relatively aseptic enclosure has been effectively used. This assay requires an incubator, plate absorbance reader (with standard filters), and a limited amount of disposables.

Assay Rationale

The traditional bacterial killing assay procedure involved growing a microbe either exposed or not exposed to sample (containing killing elements) on agar plates (Buehler et al., 2008; Matson et al., 2006; Rubenstein et al., 2008; Ruiz et al., 2010). In general the method typically requires a sample diluted in media or phosphate buffered saline added to a known number of live microbes. In short, the microbes and sample are incubated for a brief period and then added to agar plates. After a longer incubation period, microbe growth is quantified by counting the number of colonies for each sample. By comparing the sample plates to the reference plates, which have only microbes and no sample, the degree of microbial killing is determined. While fresh whole blood is preferable, field work often necessitates the use of frozen plasma. If the frozen samples are used, however, the microbiocidal capability greatly decreases with both freeze-thaws and long periods of storage (over 20 days; Liebl & Martin 2009).

It is also critical to note that this measure of immune function varies significantly among species and individuals in the same population, depending upon a variety of factors (such as sex, age, and parasite load). While this variation allows for considerable

comparison across different organisms in different contexts, it is necessary to optimize

dilutions of the sample and microbe strain prior to conducting the full assay (Buehler et

al., 2008; Matson et al., 2006; Rubenstein et al., 2008; Ruiz et al., 2010). The plating of

samples on agar plates and manually counting microbial colonies, while standard in

immunological research, is time consuming, requires comparatively large amounts of

samples, and can be less reliable. In response to these challenges, Leibl and Martin

(2009) introduced a new method that quantifies microbial colonies using a Nanodrop

spectrophotometer (Thermoscientific; Wilmington, DE). This new approach significantly

reduced variation among samples and reduced the amount of necessary sample used in

the assay. However, access to Nanodrop spectrophotometers is limited at some

institutions making it difficult to perform the assay, and the correlation between

Nanodrop and the traditional agar plate analysis is not ideal (i.e., r = 0.458), limiting its

utility as a proxy for actual bacterial killing (Liebl and Martin, 2009). Here we introduce

a new variation, the microbiocidal assay that is adapted from Liebl and Martin (2009) for

use on a microplate reader and will enable researchers across disciplines to effectively

employ this method to accurately quantify microbial killing ability, using readily

available microplate absorbance readers.

MATERIALS AND METHODS

Species selection and blood sampling

For validation of this new microbiocidal technique we chose a wide range of species across different taxa. These species were chosen because they inhabit a wide range of environmental conditions, employ different life-history strategies, are a mixture of field sampled and laboratory-housed, and have varying blood volumes. This chosen range of diversity should clearly demonstrate the flexibility and wide applicability of the microbiocidal assay.

Coyotes – Three kennel-housed coyotes (*Canis latrans*) were manually restrained and 1ml of blood was collected via the cephalic vein using a sterile 23-gauge syringe and transferred to sterile 5ml tubes.

House finches – Six wild house finches (*Carpodacus mexicanus*) were passively caught in potter traps from a site near California Polytechnic State University, San Luis Obispo, California. Thirty microliter blood samples were obtained via puncture of the alar vein with a sterile 26-gauge needle and blood was collected into microhematocrit capillary tubes, and transferred to sterile 1.5ml tubes.

Garter snakes – Thirteen laboratory-housed garter snakes (*Thamnophis elegans*) were bled via the caudal vein using sterile 26-gauge syringes. 50ul blood samples were transferred to sterile 1.5ml tubes.

Side-blotched lizards – Six individual lizards (*Uta stansburiana*) were captured via noosing and baseline blood samples of 20ul were collected from the retro orbital sinus

using a heparinized capillary tube within 3 minutes of capture. Blood samples were transferred to sterile 1.5ml tubes.

Newts – Six laboratory-housed rough skinned newts (*Taricha granulosa*), were sampled via tail snips with sterile surgical blade, 30ul of blood was then collected from the caudal vein into microhematocrit capillary tubes and transferred to sterile 1.5ml tubes.

For all above sample collections blood samples were stored on ice until further processing could take place, at which time plasma was separated from the cells via centrifugation. For all species samples of different individuals were pooled and stored at -20 ℃ until assayed.

Microbe Selection and Preparation

In the current set of validations, we used microbes *Escherichia coli* (ATCC #8739), *Staphylococcus aureus* (ATCC #6538), and *Candida albicans* (ATCC #10231). These microbes were chosen because 1) they are the most commonly used microbes in ecoimmunology studies (i.e., "the gold standards") providing abundant data for comparison (Tieleman et al., 2005; Matson et al 2006; Millet et al., 2007; Boughton et al., 2011), 2) they require different functional immune responses to kill (e.g., *E. coli-* complement dependent; *S. aureus-* complement independent, requires phagocytosis; *C. albicans-* killing is mostly by phagocytosis; Pulendran et al., 2001a; 2001b), and 3) they represent different classes of microbes and we wanted to test the range of assay applicability (i.e., *E. coli-* Gram-negative bacteria; *S. aureus-* Gram-positive bacteria; *C. albicans-* diploid fungus/yeast).

Prior to the assay, we autoclaved Tryptic Soy Broth (Sigma-Aldrich # T8907; 15g broth/500ml nanopure water) and stored it overnight at 4° C. Additionally, we reconstituted the microbes *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*, in lyophilized pellet form (Epower Assayed Microorganism Preparation) from Microbiologics Inc., Saint Cloud, MN)) in 10ml of pre-warmed 0.9% Phosphate Buffered Solution (PBS) (37° C for *E. coli* and *S. aureus* and 30° C for *C. albicans*). Using flame-sterilized forceps, we transferred the pellet to the warm PBS and vortexed the solution. We then incubated the microbe solution for 30 minutes at 37° C for *E. coli* and *S. aureus* and 30° C for *C. albicans*. Finally, we vortexed the stock solution until the pellet was completely dissolved and stored the solution for no more than 24h at 4° C. We use this stock solution to make up a working solution (10^5 colony-forming unit; CFU).

Assay Procedures

As a sterile environment was of utmost importance, we worked under an ethanol-sterilized laminar flow hood with ethanol sterilized and/or autoclaved equipment and disposables (such as pipettes, wells, and pipette tips). We thawed all of the samples, but ensured that none of the samples had previously been thawed on any occasion. All samples were run in triplicate to enable greater accuracy. For validation on new species, serial dilutions are required to obtain the optimal microbiocidal activity. Here we describe the sample volumes for the side-blotched lizard (*Uta stansburiana*).

We initially plated positive controls by adding 18 ul of PBS and negative controls by adding 24 ul of PBS only (96 well cell culture round bottom microplates). We then

added 2ul of plasma and 16ul sterile PBS (1M 10X PBS; 1:8 dilution plasma) to each of

the three wells and add 6 ul of the bacteria working solution to all wells except negative

controls. While multi-channel pipettes can be used, we strongly urge that the pipettes be

calibrated and regularly maintained to ensure accuracy when pipetting small volumes.

The plate was covered while still in the hood for the remainder of the assay. We then

vortexed the plate on a plate shaker (150 rpm) for 1 minute gently to ensure solutions are

well mixed and that there is no splashing between wells.

After vortexing, plates were incubated for 30 minutes at 37°C for *E. coli* and *S.*
aureus and at 30°C for *C. albicans*. Following incubation with microbes we again

vortexed the plate gently for 1 minute (150rpm) and added 125 ul of the sterile broth to

all wells, and included the positive and negative controls. We again vortexed (100rpm)

the plate for 1 min and read the plate using the microplate reader (BioRad xMark[TM]

Microplate Absorbance Spectrophotometer) at 300nm for *E. coli* and *S. aureus* and

340nm for *C. albicans* to determine the background absorbance. Finally, the plates were

incubated at 37°C for 12 hours or *E. coli* and *S. aureus,* and 30°C for 24 hours for *C.*
albicans.

If using whole blood - The protocol for using whole blood was very similar to

plasma except during the plating stage. We added 2 ul of the blood sample to 16 ul (1:8

dilution) of CO2-independent media plus 4mM L-glutamine (media Gibco #18045; L-

glutamine Sigma-Aldrich #G3126). For the whole blood procedure, CO2-independent

media was used instead of PBS to dilute samples. The positive and negative controls

each received 2ul PBS and 16 ul CO_2-independent media plus 4mM L-glutamine. Lastly,

we added 6 ul bacteria (prepared as described above) to each sample and positive controls. The negative controls received an additional 6 ul of PBS. The remainder of the protocol is identical to that of the plasma assay.

Reading Plate - After the sample/bacteria solution has incubated for the appropriate time (12 h for *E. coli and S. aureas* and 24 h for *C. albicans*), we used a microplate absorbance reader (BioRad xMark spectrophotometer) to read the absorbance at 300nm for *E. coli and S. aureas* and 340nm for *C. albicans* (optimized as described below).

To calculate bacterial killing ability, we first subtracted background absorbance readings from the absorbance readings (i.e., 12 and 24 hour readings). Microbiocidal capacity was calculated as one minus the mean absorbance for each sample (samples were run in triplicate), divided by the mean absorbance for the positive controls (wells containing only bacterial and broth), and multiplied by 100 (i.e., % bacteria killed relative to the positive control). The negative controls were used to ensure that there was no contamination but not used in the final calculation. Therefore, the negative control absorbance values should not vary between the background and the post-incubation read.

Optimization of bacterial growth and absorbance

Prior to testing microbiocidal ability of plasma, we optimized incubation (interval to log phase growth) and bacterial concentration. Following Leibl and Martin (2009) we used a concentration for *E. coli* and *S. aureus* of 10^4 and 10^5 colony forming units

(CFU)/ml incubated at 37˚C. Absorbance was measured at 300nm, 340nm, 405nm, 490nm, and 595nm, most of which are common filters present on most absorbance readers. We measured absorbance at 2, 4, 6, 12, 18, 24, 29, and 41 hours post-inoculation to determine log-phase growth for each bacterial species. *Candida albicans* was assessed at a concentration of 10^4 CFU/ml and was incubated at 30˚C. Absorbance (300, 340, 405, 490, and 595nm) was read at 2, 4, 6, 12, 18, 24, 29, 41, and 53 hours post-inoculation.

Optimization of different species' plasma samples

We optimized the microbiocidal assay using both *E. coli* and *S. aureus* for four different species: coyote, house finch, garter snake, and newt. This range of species should provide an approximate starting point for new researchers utilizing this technique; however, any researcher replicating this protocol should perform a species validation. To optimize for different species, we plated pooled plasma samples (3 pooled samples of 2 individuals each) for house finches, garter snakes, side-blotched lizards, and newts and individual samples (i.e., not pooled) for coyotes in the top row of 96 well microplates. We serially diluted each sample down the plate (from 1:1 – 1:128). Specifically, we added 18ul of pooled plasma sample in triplicate and 18ul PBS to the first row of the plate and then added 18ul of PBS to all other wells on the plate (except for positive and negative controls). We mixed the plasma and PBS in row 1 using a multichannel pipette. We then removed 18ul from row 1 and transfer to row 2 re-mixed the solution and repeated to each subsequent row to serially dilute down the plate (after row 8 the remaining 18ul can be disposed) for least 8 dilutions. We then followed the same assay

procedure as above. All plasma samples were incubated with bacteria (10^5 CFU/ml) for 30min at 37˚C and then for 12 hours at 37˚C following the addition of tryptic soy broth. Assay results depict average response across replicate samples for each species.

Cross-Validation

We performed simultaneous assays using equivalent sample dilutions and microbial concentrations for both the new microplate and the traditional agar plate microbiocidal analysis. We assayed 4 dilutions each of 7 different samples of *T. elegans*. Samples were not serially diluted for this validation, instead they were prepared independently. For the agar plate assay we followed the traditional, standard methodology described in French et al. (2010) and Zysling and Demas (2007). We ran a linear regression to test the new microplate microbiocidal assay against the traditional agar plate method. The significance level statistical test was $\alpha = 0.05$, and was conducted using JMP.IN (v. 8.0.1, SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Optimization of bacterial growth and absorbance

As found in previous studies, *E. coli* and *S. aureus* microbes reached log-phase growth at 12 hours of incubation at 37˚C, which is considered optimal (Fig. 1A, B). Concentrations of 10^4 CFU/ml for *E. coli* and 10^5 CFU/ml for *S. aureus* were most appropriate. Both concentrations for both microbes exhibited increasing absorbance;

however, the time course to reach log-phase growth was slightly different from 10^4 to 10^5 CFU/ml. The coefficients of variation (CVs) for the *E. coli* and *S. aureus* plates were and 0.019 and 0.016 respectively. *C. albicans* reached log-phase growth at 30 hours of incubation at 30˚C, and we tested a concentration of 10^4 CFU/ml (Fig. 1C). The CV for the *C. albicans* plate was 0.032.

Different absorbance filters were more effective at measuring microbial growth for the different microbes. For *E. coli* and *S. aureus* both 300 and 340nm filters were most optimal and for *C. albicans* a 340nm absorbance filter was best at measuring microbial growth (340nm filters are found on most standard absorbance readers).

Optimization of different species plasma samples

All species samples exhibited decreased killing with increasing dilutions, as would be expected with a serial dilution (Fig. 2A, B). It is however evident that the different species varied greatly in their killing ability among the different microbes. Researchers should therefore optimize for each individual species for each individual microbe prior to using this assay. Optimization should be for sample dilutions that yield approximately 50% killing. Therefore, higher plasma volumes than those used in the current protocol should be used when validating for new species to attain a higher percent killing.
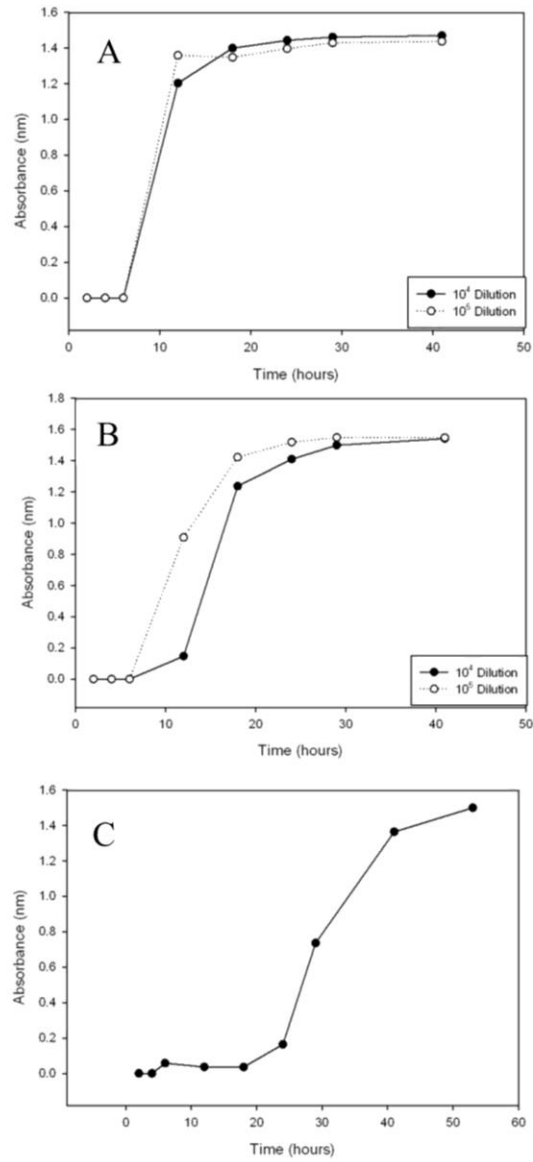
Fig. 2-1. Microbial growth measured as absorbance (nm) over time in A) *E. coli*, B) *S. aureus,* and C) *C. albicans*.
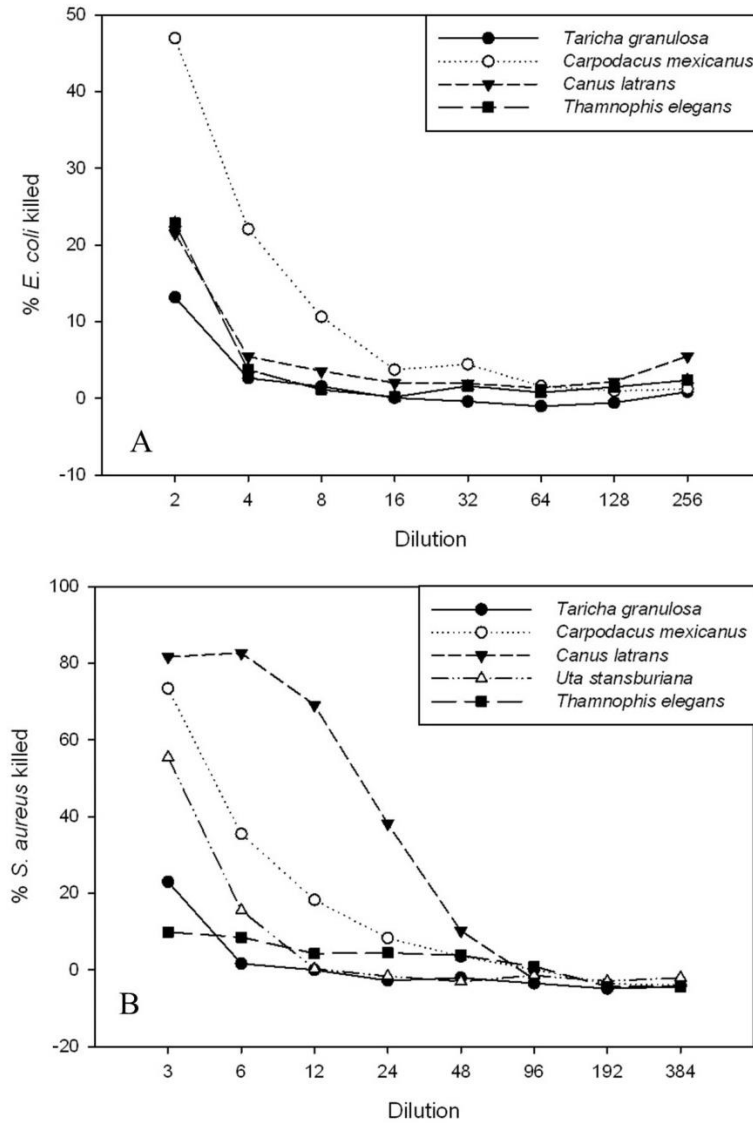
Fig. 2-2. Microbiocidal ability for **A)** *E. coli* and **B)** *S. aureus* microbes across different plasma dilutions for the non-traditional model species rough skinned newts (*Taricha granulosa;* amphibian), garter snakes (*Thamnophis elegans*, reptilian), side-blotched lizards (*Uta stansburiana),* house finches (*Carpodacus mexicanus,* avian), and coyotes, (*Canis latrans*, mammalian).
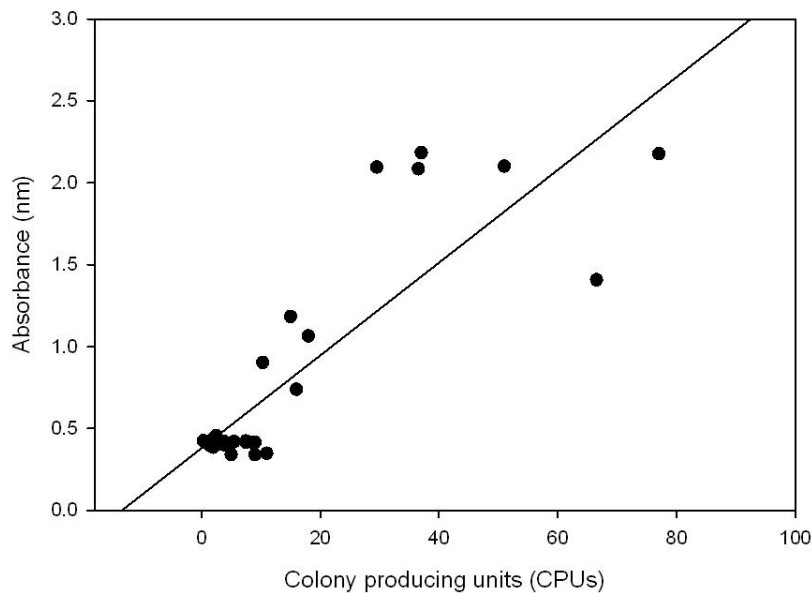
Fig. 2-3. Relationship between Microbiocidal (microplate) assay and traditional agar plate antimicrobial assessment technique using different dilutions of garter snakes (*Thamnophis elegans*) samples, tested via a linear regression (adj $R^2 = 0.71$).

Cross-Validation

The new antimicrobial microplate assay was highly correlated with the traditional agar plate antimicrobial assessment technique ($F_{1, 26} = 63.19$, $P < 0.01$; adj $R^2 = 0.71$; Fig. 3). These results suggest that the new method is a good proxy for the traditional, standard agar plate method. The fit appears best within the middle range of killing (Figure 3), and therefore the assay should be optimized (as in the traditional agar plate method) for a sample dilution that yields an average killing of approximately 50%.

CONCLUSIONS

Microbiocidal activity measured via new microplate analysis was more efficient, yielded less variation than previous methodology, and was more closely related to traditional methods of microbiocidal analysis. We hope this provides a new variation on a powerful ecoimmunological method that will enable researchers across disciplines to effectively employ this method to accurately quantify microbial killing ability.

However the microbial killing assay does not measure the immune function *in vivo* and thus requires extrapolation. Further this assay also must be optimized following similar procedures to those outlined in the species validation of this manuscript for new species and populations that are assessed in different environmental contexts, such as breeding state and time of year. Finally, samples must be centrifuged (if using plasma), frozen, and analyzed within a relatively short period of time (approximately 20-30 days to analyzing). This may pose a challenge for field researchers who do not have access to the proper equipment. In cases in the field with limited access to equipment researchers may opt for the traditional agar plate method which can be done completely in the field.

Regardless of which ecoimmunology techniques researchers choose to employ, experimental context is paramount to the interpretation of immunological data (Demas et al., 2011). Immune responses are not fixed in nature; they are instead highly variable depending on context and species (Boughton et al., 2011). Microbial killing ability may not be the best method for immune assessment for every system. However, using the microbial killing technique researchers can optimize ecological relevancy by selecting

specific microbes based on the biology of their study organism or scientific question

(Table 1). For example, is there a high incidence of a particular pathogen in the system?

Are you interested in measuring complement dependent or complement independent

immune pathways? With careful consideration for the context of the experiment and the

ecology of the organism microbiocidal activity can be a powerful and versatile tool

providing functional and relevant results.

Table 2-1. Examples of commonly used microbes for analysis of microbiocidal activity in ecoimmunology, immune responses engaged and references.

Strain	Immune response engaged	References
Bacillus subtilis ATCC #6051	Gram-positive bacteria, Complement dependent	**Nano-particles** (Wei, Sun, Qian *et al.*, 2009)
Candida albicans ATCC #10231	Diploid fungus (yeast), Killing is mostly by phagocytosis, May also rely on natural antibodies	**Birds** (Millet, Bennett, Lee *et al.*, 2007) **Plants** (Duarte, Figueira, Sartoratto *et al.*, 2005, Hammer, Carson & Riley, 1999)
Escherichia coli ATCC #8739	Gram-negative bacteria, Complement dependent, Requires phagocytosis and presence of natural antibodies	**Birds** (Millet *et al.*, 2007, Matson *et al.*, 2006) **Mammals** (Martin, Weil & Nelson, 2007)
Escherichia coli ATCC #51813	Gram-negative bacteria, Complement independent, Requires phagocytosis	**Birds** (Millet *et al.*, 2007) **Nano-particles** (Wei *et al.*, 2009, Pourjavadi & Soleyman, 2011)
Escherichia coli NCIMB 12210	Gram-negative bacteria, Complement dependent	**Fish** (Fernandes, Kemp, Molle *et al.*, 2002)
Salmonella typhi ATCC #19430	Gram-negative bacteria, Complement-dependent, Activates granulomas, Requires presence of natural antibodies	**Mammals** (Tagliabue, Villa, Boraschi *et al.*, 1985)
Staphylococcus aureus ATCC #6538	Gram-positive bacteria, Complement independent, Requires phagocytosis and presence of natural antibodies	**Birds** (Millet *et al.*, 2007b, Matson *et al.*, 2006)
Staphylococcus aureus ATCC #27661	Gram-positive bacteria, Complement independent, Requires phagocytosis	**Nano-particles** (Wei *et al.*, 2009, Pourjavadi *et al.*, 2011)

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CHAPTER 3

WOUND HEALING REDUCES STRESS-INDUCED IMMUNE CHANGES:

EVIDENCE FOR IMMUNE PRIORITIZATION IN THE SIDE-BLOTCHED LIZARD[2]

Immune system function is affected by a variety of exogenous and endogenous stressors. Most studies have focused on the effect of stressors on immune function, but not necessarily on trade-offs within the immune system and interactions with energy-mobilizing hormones. In this study, we examined how bactericidal ability and corticosterone interacted by applying acute restraint stress in a non-model organism, the side-blotched lizard (*Uta stansburiana*), ten days after receiving a cutaneous wound. We found a decrease in bactericidal ability in wounded animals after restraint stress. However, the percentage healed during the first seven days was positively correlated with bactericidal ability ten days after wounding. Additionally, the magnitude of change in corticosterone concentration during the acute stress was positively correlated with the percentage of wound healing during the first three days. These two relationships may demonstrate a "faster is better" strategy. If energy is invested heavily in the initial wound healing stages, the individual may be able to mount a more effective immune and stress response earlier.

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[2] Coauthored by Lorin A. Neuman-Lee and Susannah S. French. Reprinted with permission from. Journal of Comparative Physiology B, pages 1-7, 2014.

INTRODUCTION

Wound healing is an integrative and highly relevant immune response that requires the interaction among the different branches of the immune system (innate, humoral, and cell-mediated; Demas et al. 2011; Janeway et al. 2001; Martin 1997). Healing a wound is a complex and energetically expensive process that involves multiple stages, including an initial inflammatory response, the recruitment and proliferation of cytokines, platelets, chemokines, and granulocytes, and finally granulation and remodeling of the wound site (Lochmiller and Deerenberg 2000, Werner and Grose 2003). This process is biologically relevant to all vertebrates because cutaneous injuries are common occurrences (French et al. 2008). However, healing a wound is complicated by the fact that wild organisms must also invest limited resources into other energetically demanding processes, including exogenous (coping with weather changes, predation attempts, social interactions) and endogenous (reproduction and self-maintenance) stressors (French et al. 2007a; Wingfield 2005). In the context of stress physiology, the response of wound healing is often examined in the presence of these additional stressors as a measure of self-maintenance, but not as a distinct stressor (Padgett et al. 1998; French et al. 2006).

Evidence from stress physiology studies has demonstrated that stress from both exogenous and endogenous stress factors can alter the rate at which a wound is healed. This is likely due to the mobilization of energy by glucocorticoid (GC) hormones away from nonessential physiological processes to processes required for immediate survival

(Wingfield and Romero 2011; Sapolsky et al. 2000). These GCs, including corticosterone (CORT) in reptiles, birds, amphibians, and fishes, are released through the activation of the hypothalamic-pituitary-adrenal (HPA) axis and are likely the primary mediators of energetic trade-offs (French et al. 2007b; Sorrells and Sapolsky 2007). Glucocorticoids exert a myriad of effects on multiple physiological systems, many of which can directly affect wound healing (Sapolsky et al. 2000).

However, the effects of elevated GCs are highly context-dependent and are affected by the magnitude and duration of the elevation and the energetic state of the individual (Brooks and Mateo 2013; French et al. 2007; Moore and Jessop 2003; Angelier and Wingfield 2013). For example, acute elevation of CORT is generally considered to be beneficial to an individual due to the activation of the immune system, mobilization of energy from nonessential to essential processes, and redistribution of immune cells (McEwen 2007; Sorrells and Sapolsky 2007; Dhabhar and McEwen 1997; Dhabhar et al. 2012; Hopkins and DuRant 2011). Conversely, chronic elevation of CORT typically causes negative effects on the organism (Wingfield 2005; Dhabhar 2009a; Dhabhar 2009b), including suppressed immune function (Dhabhar 2009a; Dhabhar 2009b), inhibited reproductive output (Moore et al. 2005), muscle catabolism (Marra and Holberton 1998; Hasselgren 1999), and potentially even death (Selye 1946). Because of these effects, understanding the role of GCs in wound healing in the absence of additional stressors is critical if we are to examine GCs within the context of other stress factors.

This need is reinforced by the fact that receiving and coping with a wound is likely a stressor in itself and may cause an elevation of GCs even in the absence of other stressors. Early stages of wound healing initiate the inflammatory response, which activates innate immune components, such as macrophages, heterophils, neutrophils, and the complement system (Demas et al. 2011). This response is also activated during a bacterial infection, and therefore the efficacy of this response can be assessed in conjunction with wound healing to determine the allocation of immune cells at the wound site and in the circulating plasma (Demas et al. 2011; French and Neuman-Lee 2012).

Additionally, much of our understanding of the immune and stress physiology arises from using avian and mammalian models (Matson et al. 2006; Janeway et al. 2001). In contrast, reptilian models are little studied, despite unique immune strategies and an important place in evolutionary history (Zimmerman et al. 2010). Wound healing has been examined histologically in lizards and snakes and, although the progression of healing is consistent with other models, key differences exist. Due to the thin dermis in snakes and lizards, dermal repair primarily occurs horizontally while mammalian repair typically occurs vertically through the subcutaneous tissue (Maderson and Roth 1972; Smith and Barker 1988). The other key difference between mammalian/avian models and reptilian models is in the context of their inflammatory response, albeit in distinct ways. For example, in snakes, there is a greater density of heterophils (analogous to neutrophils in mammals) than macrophages in fibrin-containing areas and a disappearance of macrophages early in the healing process (Smith and Barker 1988), which differs greatly from the mammalian response. However, while the snake response is extremely robust,

lizard wounding may result in a minimal inflammatory response (Smith and Barker 1988; Maderson and Roth 1972). The marked differences between reptilian responses and mammalian/avian responses, as well as between the two reptiles, illustrate the important need to examine how different immune responses interact in a variety of taxa.

Therefore, the purpose of this study was to assess stress physiology during wound healing and to examine how wound healing affects innate immune function in the absence of other stressors. Additionally, we wanted to measure these interactions in an organism that relies heavily on wound healing in nature, the side-blotched lizard, *Uta stansburiana*. To accomplish this, we gave an experimental wound to one group of lizards and, after ten days, applied an acute experimental stressor to determine the lizards' ability to mount a stress response. We measured circulating levels of CORT and bactericidal ability for baseline and stress-induced samples and correlated the rate of healing to CORT and bactericidal ability. We predicted that 1) the wounded lizards would have decreased bactericidal ability and CORT after the acute stress challenge, and 2) the rate of healing would be negatively correlated to CORT levels and positively related to bactericidal ability.

METHODS

Animal collection and care

The side-blotched lizard (*Uta stansburiana*) is a well-studied, short-lived lizard with a wide geographic range (Tinkle 1967; Parker and Pianka 1975; Lucas and French

2012). We collected 26 female adult lizards in May of 2011 near St. George, UT by noosing. We transported the animals back to Utah State University immediately where they were housed individually in plastic terraria with a newspaper substrate, a water bowl, and refugium for 1 month prior to the experiment. Each cage was equipped with temperature-controlled heat tape and an ultra-violet light on a 12:12 cycle. We fed all lizards two-week old crickets (Flukers Farms) *ad libtum* throughout the acclimation and experimentation period. Females were in early or mid-vitellogenesis (determined by palpation), but reproductive state was not statistically significant in any measurement. All experimental procedures were approved by the Utah State University Institutional Animal Care and Use Committee (Protocol #1149).

Wound Healing

We randomly assigned all 26 animals to either the wound (n = 14) or control sham-wound (n = 12) treatment group. Following the methods outlined in French et al. (2006), we anesthetized the lizards using surface-induced hypothermia. Animals were either given a 3.5 cm biopsy on the dorsal side of the lizard between the hind legs using a sterile biopsy punch, or underwent a sham biopsy surgery, which consisted of anesthetizing the lizard and pressing on the dorsal side with the blunt end of the biopsy punch. A digital image of all animals (wounded and non-wounded) was taken using a Pentax K-x digital camera. Every 3 days, we took a digital photograph to document the rate of healing to create 3 more time points. On day 10, we took the final image. All non-wounded animals were also photographed in this manner to maintain consistency.

Images were analyzed blindly by randomizing images and measuring them using image analysis software (Image J, NIH Imaging). The area of the wound was calculated at each time point for each individual and the percent of wound healed between each time point was determined. We excluded one individual after Day 4 and four individuals after Day 7 due to either the regenerating tissue peeling off or an unclear photograph.

Blood collection

Because of the small size of *U. stansburiana*, we were only able to conduct a stress-reactivity test (baseline and stress-induced sample) one time throughout the wound healing process. Thus, we took a baseline and stress-induced sample ten days after the initial wounding. Another study on small lizards indicates that ten days post-wounding provides a clear healing profile and detectable changes in CORT and innate immune activity (French et al. 2006). We removed each lizard individually from its cage and, within three minutes, obtained a blood sample via the post-orbital sinus using a heparinized capillary tube (50 µl). We collected one capillary tube per sample. We placed each lizard individually in a cloth bag for 10 minutes, after which we took another blood sample. Each blood sample was collected into a 1.5 ml Eppendorf tube and placed on ice for less than 1 hour until we centrifuged all samples at 2200 rpm for 10 min at 4°C. We separated the plasma from the red blood cells and stored the samples at -20°C until further analyses were conducted.

Bactericidal Assay

We performed the bactericidal assay following the protocol outlined in French and Neuman-Lee (2012). Briefly, we combined a 1:12 plasma dilution with CO_2-Independent media (Gibco, Grand Island, NY) plus 4nM L-glutamine (Sigma-Aldrich), and 10^5 CPU (colony producing unit) *Escherichia coli* (EPowerTM Microorganisms #0483E7, ATCC 8739, MicroBioLogics, St. Cloud, MN), and agar broth on a 96-well microplate. We calculated the background absorbance using BioRad xMark microplate reader. After a 12-hour incubation, we again read the absorbance and calculated the bactericidal ability by dividing the mean absorbance for each sample (run in duplicate) by mean absorbance for the positive controls (containing only media and bacterial solution), and multiplying by 100. This provides the percent bacteria killed relative the positive controls. Negative controls (containing media only) were also run to ensure that there was no contamination.

Radioimmunoassay

Circulating corticosterone concentrations were determined using a protocol modified from Moore (1986). Briefly, we performed extractions using a solution of 30% ethyl acetate: isoocation. We snap-froze the samples using a mixture of methanol and dry ice, poured off the supernatant and evaporated the remaining solvent with N_2 gas. We then resuspended the sample using phosphate-buffered solution (PBS) and assayed in duplicate for CORT (Fitzgerald 20-CR45, Lot #P0012502). We determined the individual recoveries using a separate aliquot of the resuspended fractions. The

recoveries account for any potential loss during the assay and allowed us to adjust the final sample concentration. Inter-assay variation was 11.2%.

Statistics

All measures were analyzed for assumptions of normality. Corticosterone concentration was log-transformed to meet these assumptions. We were unable to transform bactericidal ability to meet these assumptions, so we used nonparametric analyses. We assessed the effect of treatment and time using a repeated measures analysis of variance (rmANOVA) using the baseline and stress-induced samples as our time points. We also tested the effect of treatment on the baseline and stress-induced sample independently using a t-test for CORT and a Wilcoxon Rank-Sum Test for bactericidal ability. Because we are unable to truly determine the independent variable, correlational analyses were conducted between each measure of bactericidal ability (Baseline, Stress-induced, and Delta) and each measure of CORT concentration (Baseline, Stress-induced, and Delta). Finally, the percent healing between each of the pairs of time points (Day 1-4, Day 1-7, Day 1-10, Day 4-7, Day 4-10, Day 7-10) was correlated to each of the six measures of bactericidal ability and CORT. All analyses were run in JMP 10.0.2 (SAS Institute, Inc., 2012).

RESULTS

Bactericidal Ability

Individuals that underwent wound healing had a lower bactericidal ability after the stress challenge at the end of the experiment compared with the unwounded animals ($X^2 = 4.56$, p = 0.03; Figure 1). There was no difference between the baseline bactericidal ability of wounded and non-wounded animals ($X^2=0.002$, p = 0.96). There was no change between baseline and the stress-induced bactericidal ability (delta; $X^2=1.87$, p = 0.17) between wounded and non-wounded animals. In our repeated measures model, we found no relationships between the baseline and stress-induced samples, the treatment, or the interaction between the two (p > 0.1). Finally, we found no correlations between any measure of bactericidal ability and CORT concentrations (baseline, stress-induced, delta; p > 0.2).

Corticosterone Concentrations

We determined that there was an overall increase in CORT concentration for all individuals between the baseline and stress-challenge bleeds ($t_{(26)} = 3.16$, p = 0.003), regardless of treatment group. There was no effect of wounding on baseline, stress challenge, or the difference between the two (p > 0.1; Figure 2). While the stress-induced sample was dependent upon the baseline sample (p = 0.035), there was no relationship in

the repeated measures analysis between CORT levels and treatment or time x treatment

(p > 0.1).

Wound Healing

The wound healing profile was similar for all wounded animals (Figure 3), with

the greatest healing taking place during the first time period (Day 1-4). We found that the

percentage of wound healed between Day 1-7 was positively correlated with stress-

induced bactericidal ability (R^2=0.56, p = 0.043; Figure 4).
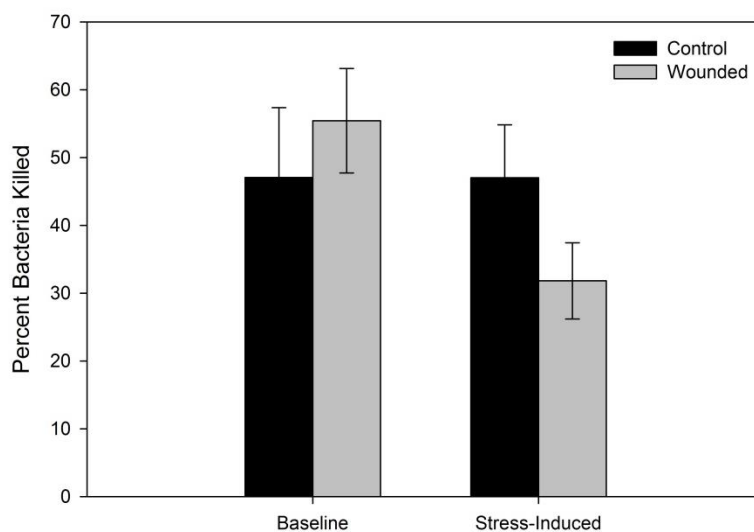
Fig. 3-1. Percent of *E. coli* killed in samples from side-blotched lizards (*Uta stansburiana*) with either a cutaneous wound (n = 14) or a sham wound (n = 12). While the baseline samples were not different between the two treatment groups (p > 0.2), the wounded individuals had a significantly lower bactericidal ability after being subjected to the stress challenge (p = 0.03). The clear bars indicate the baseline bactericidal ability and the shaded bars indicate the sample taken after a 10 minute acute stress. Error bars indicate ± 1 standard error.

We distinguished a positive relationship between percentage healed during Day 1-4 and the delta CORT level between baseline and stress-induced bleeds ($X^2 = 0.54$, $p = 0.041$; Figure 5). The percentage of wound healed during any of the other time periods was not related to any other baseline or stress-induced measures of CORT or bactericidal ability ($p > 0.1$).
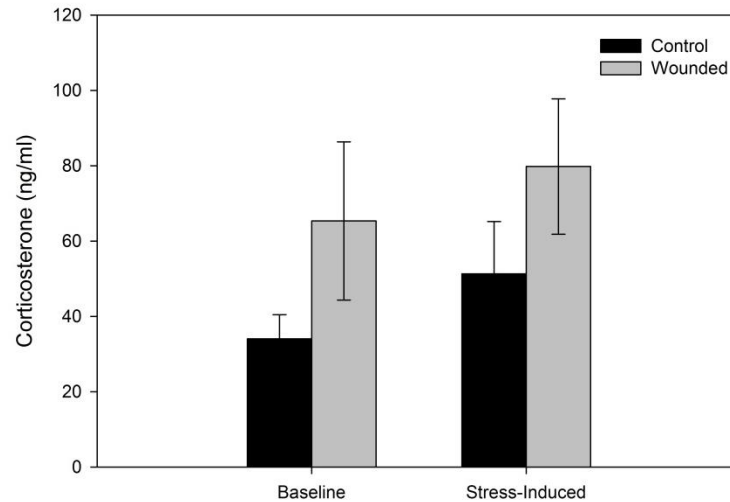
Fig. 3-2. Corticosterone concentrations (ng/ml) of both the baseline and stress-induced samples in side-blotched lizards (*Uta stansburiana*) are not significantly different 10 days after receiving a cutaneous wound (n = 14) or a sham wound (n = 12; p > 0.1). Open bars indicate the baseline sample, grey bars indicate the 10-minute acute stress sample. Error bars indicate ± 1 standard error.
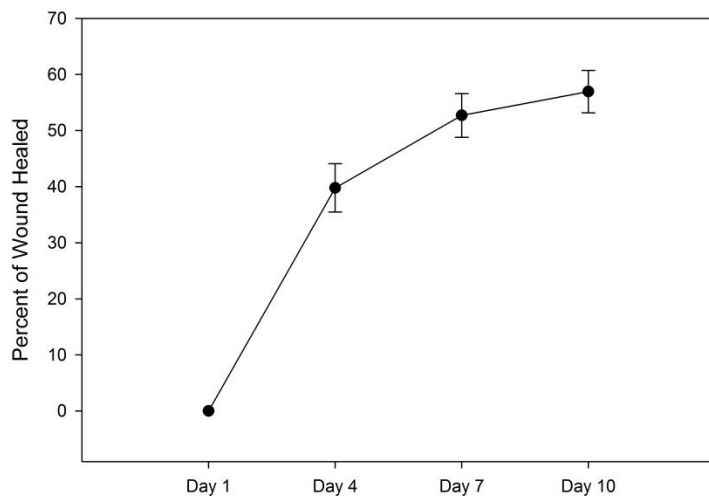
Fig. 3-3. Found healing profile in side-blotched lizards (*Uta stansburiana*) given a cutaneous wound on their dorsal surface between the hind legs.
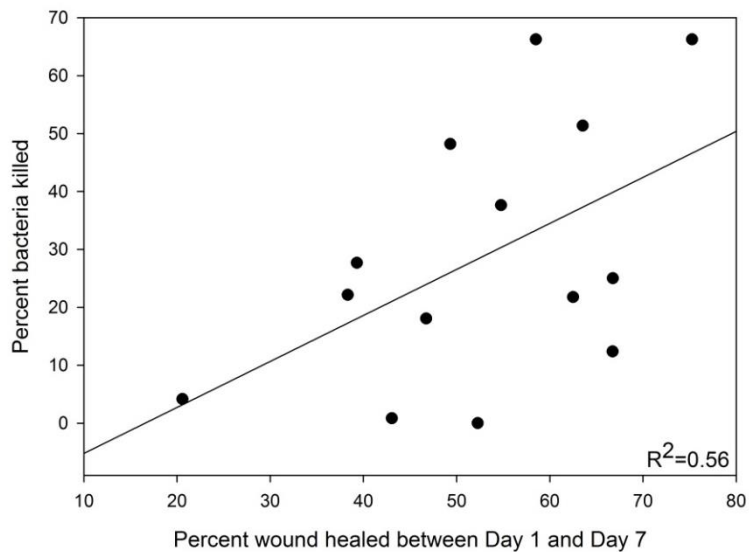
Fig. 3-4. Percent of cutaneous wound healed during the first 7 days is positively correlated with bactericidal ability 10 days after the initial wound (p = 0.043) in side-blotched lizards (*Uta stansburiana*).
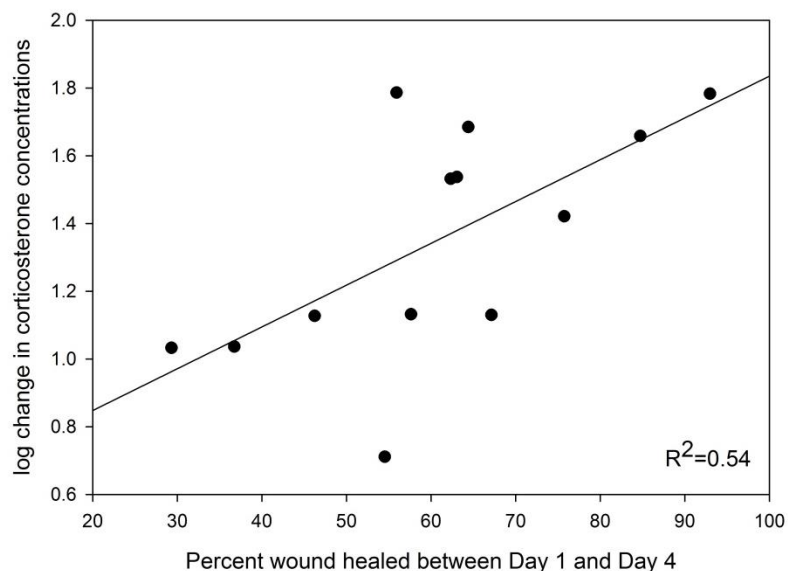
Fig. 3-5. Percent of cutaneous wound healed during first 3 days is positively correlated with the magnitude of the stress response (change from baseline to 10-minute stress bleed) as measured by corticosterone in side-blotched lizards (*Uta stansburiana*; p = 0.041).

DISCUSSION

Overall, wounded animals prioritized cutaneous healing over bactericidal capabilities, whereby wounded animals had an inhibited innate immune response after being presented with an acute stress-challenge ten days after the initial wound. However, a higher bactericidal ability was also associated with a greater early rate of healing, suggesting that some animals perform better in all immune capacities than others. Additionally, in wounded animals corticosterone responsiveness was related to early immune-regulated healing rates, although there were no differences in either baseline or

stress-induced CORT levels among wounded and sham animals at ten days. Moreover, the fact that the animals that were better able to mount a stress response healed at quicker rates during the initial stages of healing suggests that they were better able to mobilize resources toward healing.

The reduced bactericidal ability after ten days may be mediated by one or both of two mechanisms: 1) the immune cells have aggregated predominately at the wound site and are no longer circulating in the blood stream (Dhabhar et al. 2012) and/or 2) total immune function is decreased due to increased production of CORT (Sapolsky et al. 2000). A third hypothesis is that an energetic trade-off between responding to the stress and mounting an immune response resulted from limited resource availability (French and Moore 2008). This is unlikely in this study given that all animals were fed *ad libtum* and were therefore not energetically compromised. However, food consumption was not measured and therefore cannot be excluded.

The first hypothesis states that there is not a decrease in the overall number of immune cells in the body, but rather a decrease in circulating immune cells due to the aggregation of these cells at the wound (Dhabhar et al. 2012). The action of these redistributing cells, such as monocytes, leukocytes, and natural killer cells, is measured in the bactericidal assay we conducted (French and Neuman-Lee 2012). Other studies that support this hypothesis show a significant increase in these immune cells during the first 30 minutes after the acute stress, followed by a decrease as these cells migrate to the site of injury or perceived injury (Dhabhar et al. 2012). While this time frame could potentially contradict our results, it was constructed using a mammalian model and may

not be directly applicable in a reptile system. Indeed, evidence in snakes shows that heterophils were present in a healing wound until the maturation of epithelium (Smith and Barker 1988) and heterophils persisted for 117 days during an inflammatory response, which is much longer than persistence in mammals (Tucunduva et al. 2001).

The second hypothesis posits that there is an increase in glucocorticoids due to the stress of receiving the wound. Studies have correlated decreased wound-healing ability and bactericidal ability with increased levels of glucocorticoids (Rojas et al. 2002). The decrease in immune function is likely mediated by CORT, because this adrenal steroid has been shown to inhibit cytokines (Dobbs et al. 1996) and decrease immune cells in circulation (Dhabhar et al. 1995). We did not observe a difference between CORT concentrations in the wounded and non-wounded animals. However, Hawes et al. (1995) found that while an injury caused an acute increase in CORT concentrations in rats, the elevation of CORT was not sustained throughout the entire healing period.

Lizards that healed faster in the initial healing stage (Day 1-4) had a higher magnitude of CORT increase during the stress-challenge, although the absolute levels of CORT were not different between wounded and non-wounded animals. Interpretation of differences in levels of stress-related hormones is increasingly controversial, and understanding the role of downstream physiological processes such as immunity is thus critical (Romero 2004; Breuner et al. 2013; Breuner et al. 2008). However, in reptiles the change in CORT from baseline to stress-induced levels is more consistently affected by external stressors than either baseline or stress-induced absolute levels (Lucas and French 2012; French et al. 2010). The fact that the wound healing rate during this critical period

was so closely associated with the stress reactivity challenge ten days after wounding indicates that at least the initial immune stages of wound healing are stress-related and that wounded animals were undergoing physiological and/or energetic stress.

Initial healing largely dictates the later stages of healing of a cutaneous wound (Hübner et al. 1996). This finding demonstrates that lizards that were able to mount an immune response quickly and efficiently were also able to invest more energy in an acute stress response. Elevated CORT levels are consistent with a decreased rate of healing (Padgett et al. 1998; Ebrecht et al. 2004) and suppression of cytokines such as tumor necrosis factor alpha (TNF-α) and interleukins 1 alpha and beta (IL-1α and IL-1β; Hübner et al. 1996). This initial healing period shows the most pronounced decrease in wound size (French et al. 2006) and the highest level of pro-inflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-6, IL-8) is seen during the first 12-24 hours (Hübner et al. 1996; Christian et al. 2007). Therefore, the lizards that healed more quickly during this very energetically costly stage were potentially able to regain energetic stores in order to mount a more robust response after ten days.

The positive relationship between wound healing rate and bactericidal ability also supports the idea that "faster is better." The innate immune cells measured by the bactericidal assay likely aggregated around the wound, and as it healed, returned to circulation. If the wound healed faster, the cells can enter back into circulation faster and thus improve the plasma's bactericidal ability.

While the effects of stress on immune function are well-documented, the functional interactions between wound healing, innate immune function, and

glucocorticoid-mediated trade-offs remain to be elucidated. This is especially important because the scenario of multiple concurrent immune responses, especially wound healing and bactericidal activities, is realistic for many wild species. This study adds greatly to our understanding of how reptiles respond to acute stressors during the healing process and indicates that investing a high amount of energy in healing the wound initially may have benefits in a longer time frame.

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CHAPTER 4

THE ROLE OF CORTICOSTERONE AND TOXICITY IN THE ANTIPREDATOR

BEHAVIOR OF THE ROUGH-SKINNED NEWT (*TARICHA GRANULOSA*)[3]

A variety of mechanisms are responsible for enabling an organism to escape a
predatory attack, including behavioral changes, alterations in hormone levels, and
production and/or secretion of toxins. However, these mechanisms are rarely studied in
conjunction with each other. The Rough-skinned Newt (*Taricha granulosa*) is an ideal
organism to examine the relationships between these mechanisms because their
behavioral displays and toxin secretion during a predator attack are well documented and
readily characterized. While we found no direct relationship between antipredator
behavior and endogenous levels of corticosterone (CORT), antipredator behavior was
inhibited when exogenous CORT and adrenocorticotropic hormone (ACTH) were
administered, resulting in high circulating concentrations of CORT, indicating that CORT
may play a role in mediating the behavior. There was no correlation between the animal's
toxicity and either CORT or behavior. The results of this study provide evidence that
CORT plays an important, yet complex, role in the antipredator response of these
amphibians.

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INTRODUCTION

Perceiving predators and escaping predator attacks are considered highly stressful events and typically result in the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Cockrem and Silverin, 2002; Figueiredo et al., 2003). During this activation, glucocorticoids (GCs) are released into the blood stream from the adrenal glands. These GCs direct energy away from non-essential life-processes, such as reproduction, and mobilize energy stores in order to promote survival (Sapolsky et al., 2000). Often the intensity and duration of acute stressors can be gauged by the concentration of the glucocorticoid, corticosterone (CORT; Friedman et al., 1967). This release of CORT is considered to both aid in immediate survival as well as prepare for future stressful events, especially predator attacks (Wingfield, 2005).

While predator stimuli, such as visual (Cockrem and Silverin, 2002; Thaker et al., 2009a), olfactory (see review in Apfelbach et al., 2005; Fraker et al., 2009), or a combination of stimuli (Figueiredo et al., 2003), are a well-established sources of stress, the interaction between antipredator behavior and the role of CORT has been poorly studied and provides conflicting evidence. In some cases, an acute increase in circulating CORT is associated with a corresponding increase in antipredator behavior. For example, increased acute CORT concentration enhances antipredator behavior in tree lizards (Urosaurus ornatus; Thaker et al., 2009a, b) and marine iguanas (*Amblyrhynchus cristatus*; Berger et al., 2007). Conversely, experimentally increased maternal CORT

concentration in snakes reduced antipredator behavior in offspring (Robert et al., 2009).
Further, when CORT levels were experimentally elevated using dermal patches in both
Red-legged salamanders (*Plethodon shermani*; Wack et al., 2013) and Allegheny dusky
salamanders (*Desmognathus ochrophaeus*; Ricciardella et al., 2010), there was no change
in locomotor behavior, which can be considered antipredator behavior.

When CORT synthesis is inhibited using drugs, such as metyrapone, which
blocks the 11β-hydroxylation reaction (Dixon et al., 1985), the relationship between
antipredator behavior and the possible mediator corticosterone becomes even more
complex. While Thaker et al. (2010) found that application of metyrapone decreased
antipredator behavior in lizards, Hossie et al. (2010) discovered no change in behavior
associated with metyrapone's suppression of CORT in frogs. Therefore, utilizing
metyrapone can help determine whether CORT is the primary contributor to controlling
the behavior or if upstream/related hormones, such as adrenocorticotropic hormone
(ACTH) or corticotropin releasing hormone control the behavior instead (Moore and
Miller, 1984).

To further understand the role of CORT in behavior, application of the upstream
hormone ACTH can be used to test adrenal sensitivity. There is a complex negative
feedback loop and signaling, which regulates the secretion of CORT and resulting
behavioral responses (Sapolsky et al., 2000). Therefore, targeting different points in this
system will provide a more comprehensive understanding of the relationship between
CORT and behavior. ACTH, secreted by the pituitary gland, directly stimulates the
adrenal glands to secrete CORT (Wingfield, 2005).

Although amphibians have been the subject of a variety of studies examining the interaction between CORT concentrations and stressors, such as food restriction (Crespi and Denver, 2005), contamination (Hopkins et al., 1997), and captivity (Coddington and Cree, 1995), activation of the HPA axis due to predation as a potential stressor has only been examined in a handful of species, none of which produce potent antipredator toxin (Bliley and Woodley, 2012; Fraker et al., 2009; Hossie et al., 2010; Moore et al., 2005; Moore and Miller, 1984; Ricciardella et al., 2010; Wack et al., 2013). Amphibians provide an excellent model to analyze this interaction given that there are clearly defined and serialized behaviors associated with antipredator behavior (Brodie, 1977; Brodie et al., 1974).

The Rough-skinned Newt (*Taricha granulosa* Skilton; Caudata: Salamandridae) is a common caudate amphibian on the west coast of North America that utilizes two classic mechanisms for avoiding predation (Johnson and Brodie, 1975). They secrete a highly toxic substance, the neurotoxin tetrodotoxin (TTX), and exhibit this toxicity through aposematic coloring on their ventral side (Brodie, 1968). To display this coloring, newts utilize the unken reflex, in which their head and tail curve upwards and often touch each other (Figure 1). This behavior effectively shows the majority of the ventral surface, and is instigated by predation or the perception of predation (Johnson and Brodie, 1975). Newts have highly variable levels of TTX (Hanifin et al., 1999), but the relationship between individual or population toxicity and the unken reflex has not been examined.
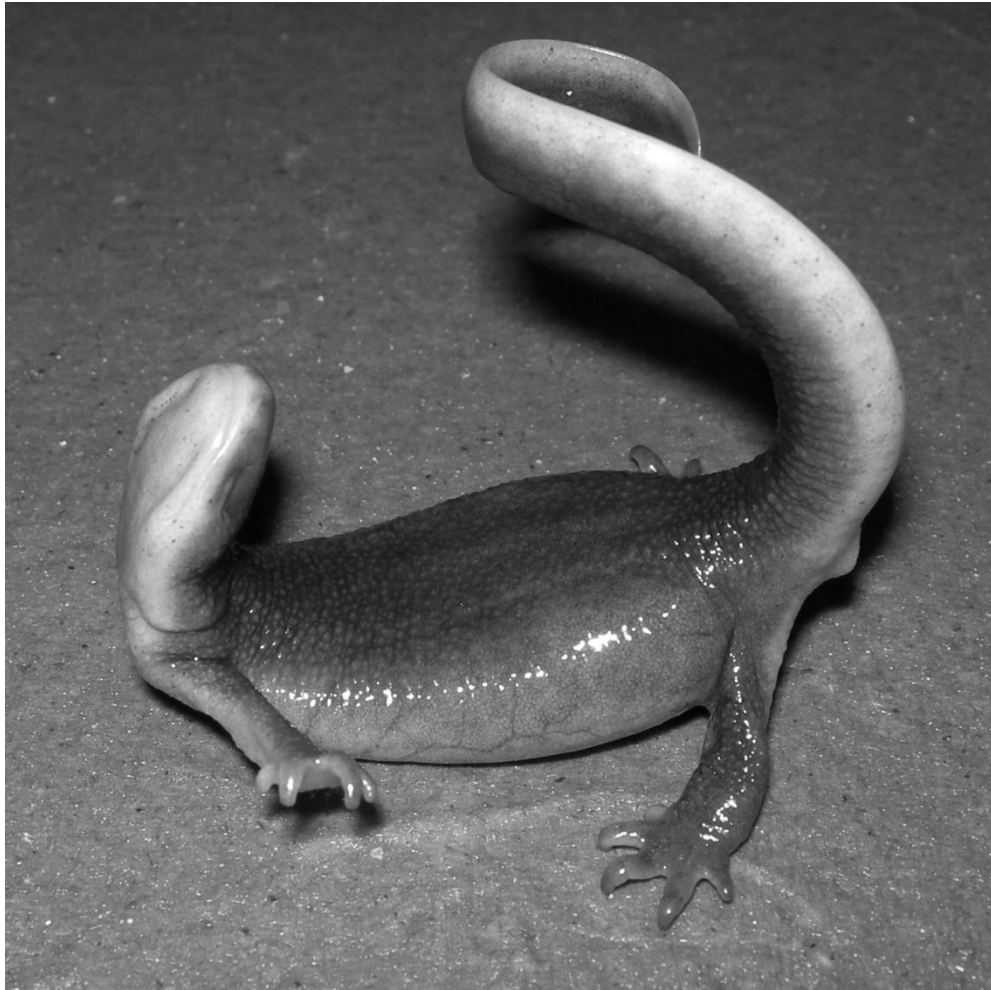
Fig. 4-1. Antipredator behavior of the Rough-skinned Newt (*Taricha granulosa*). This behavior, known as the unken reflex, allows the newt to display its bright orange ventral surface. Photograph by Edmund D. Brodie, Jr.

Tetrodotoxin is a highly potent, low molecular weight neurotoxin (Mosher et al., 1964), which inhibits propagation of action potentials by blocking the pore region of voltage-gated sodium channels in nerve and muscle tissue (Narahashi et al., 1967). TTX is found in a wide array of taxa, including both vertebrate and invertebrate groups (reviewed in Chau et al., 2011; Miyazawa and Noguchi, 2001). Though the ecological

functions of TTX seem to be wide-ranging (Hwang et al., 2004; Ito et al., 2006; Matsumura, 1995; Zimmer et al., 2006), many species utilize TTX as an antipredator mechanism (Brodie, 1968; Williams et al., 2010). While the evolutionary origins of TTX are currently debated (e.g. Hanifin et al., 2002; Noguchi et al., 1986; Shimizu and Kobayashi, 1983), levels of this toxin are highly variable across populations (Hanifin et al., 2008) and ontogenetic stages (Gall et al., 2011). Theoretical and empirical data have shown positive relationships to aposematic coloration intensity and toxicity (Blount et al., 2009; Maan and Cummings, 2012). Behavioral signal (such as the unken reflex) duration or intensity have been examined, but may demonstrate a similar relationship.

We tested the interaction among different phases in the HPA axis response, the unken reflex duration, and basal TTX levels. We hypothesized that increased unken reflex time would result in a higher concentration of CORT because the newt felt threatened for a longer period of time. We hypothesized newts would display honest signaling, thus the duration of unken reflex would be positively related to the amount of basal levels of TTX. Additionally, we predicted that endogenous CORT levels and adrenal sensitivity (measured by CORT levels after ACTH injection) would be related to unken reflex time. To test these hypotheses, we conducted three experiments: 1) compare CORT levels in animals experiencing a simulated predator attack (stimulated) to animals only experiencing handling in the absence of predator simulation (control), 2) in stimulated animals, measure relationships between endogenous levels of CORT, time spent in unken, and TTX levels, and 3) determine endocrine mechanisms involved in

antipredator behavior by injecting exogenous CORT, ACTH, or metyrapone and measuring endogenous CORT levels and time in unken.

MATERIALS AND METHODS

Study animals

Newts were collected by hand, dip net, and minnow trap from Benton and Curry Counties, Oregon, USA. All newts were housed individually in temperature and light-controlled chambers (14ºC; 12 hr on:off cycle). Newts were weighed to determine body condition. For all experiments, all individuals were females in non-breeding condition. For experiment 1A and 1B, we randomly assigned 35 newts to two different treatment groups. Ten animals received no predator simulation, while 25 animals were subjected to dorsal stimulation (described below). Animals in experiment 1 were held in captivity for > 1 year. For experiment 2, we randomly divided a separate set of 77 individuals into one of four treatment groups (described below). Animals in experiment 2 were held in captivity for 9 months prior to testing. All procedures were approved by Utah State University IACUC (Protocol #1524).

Experiment 1A: Relationship between CORT and stimulation

We removed all of the newts from the temperature-controlled chamber and conducted the experiments in a separate laboratory (approximately 24°C). To ensure that any change in CORT was associated with predator stimulation and not associated with

removing newts from their chamber and handling them, we divided the first set of

animals into a control group (n = 10) that were removed from their enclosure but not

stimulated (described below in *Behavioral analysis*) and an experimental group (n =25).

Regardless of whether or not the animal was stimulated, blood samples were taken 30

min after removal from the chamber and analyzed (collection and analysis described

below).

Behavioral analysis

Newts were stimulated (within one minute of removing animal from chamber) to

enter the unken position by light tapping of forceps mid-dorsally to simulate a bird

predator attack (Johnson and Brodie, 1975). We documented the time from the newt's

initiation of the unken posture to the time it left the unken posture (determined by the

newt taking one step with a front foot). Regardless of how long the newt remained in

unken, we took a blood sample 30 minutes after initial stimulation via the caudal vein.

Heparinized capillary tubes were used to collect approximately 40 μl of blood. All of the

blood samples were immediately centrifuged to separate the plasma and red blood cells.

The samples were stored at -20°C until further analysis (see below).

Hormone analysis

For all experiments, we quantified circulating hormone levels using a previously

described and established radioimmunoassay (RIA) protocol with a slight modification

(French et al., 2010). Extractions were performed using a solution of 30% ethyl acetate:

isooctane. Following extraction, we dried the samples, resuspended them in PBS buffer, and assayed in duplicate for CORT (H[3] Fitzgerald 20-CR45, Lot # P0012502; Antibody—MP Biomedicals, LLC, Lot 3R3-PB). Antibody cross-reactivity for corticosterone is 100%, with the next highest cross-reactivity occurring at 2.3% with desoxycorticosterone. All other cross reactivities are < 0.05%. For each sample we used an aliquot of the resuspended fractions to measure individual recoveries following extraction and chromatography. These recoveries are used to adjust the final sample concentration values to account for any losses during these procedures. Interassay variation was 11.6% and precision was 93.6%.

Experiment 1B: TTX toxicity, endogenous CORT concentrations, and time in unken

<u>*Tetrodotoxin analysis*</u>

For the 25 stimulated and 10 non-stimulated animals, skin biopsies for quantification of TTX were taken following established methods for TTX quantification (Hanifin et al., 2002). Newts were anesthetized using 1% ms222 (Tricaine-S, Western Chemical Inc., Ferndale, WA) to take 3 mm skin-punch biopsies. All biopsies were taken at least one month prior to any experimental trials described here and all wounds were completely healed at time of trials. Tetrodotoxin was extracted from skin tissues using the methods of Hanifin et al. (2002). Quantification of TTX was done using a Competitive Inhibition Enzymatic Immunoassay (Stokes et al., 2012). We used the linear range of the standard curve, which was between 10 and 500 ng/mL, to calculate sample values. Samples were diluted 1:120, 1:300, 1:500, 1:800 or 1:1000 in a 1% Bovine Serum

Albumin-PBS solution (BSA) to get them within the linear range of the standard curve. These samples were run against standards also diluted in BSA. Whole newt TTX was calculated using the methods of Hanifin et al. (2002). The average intra-assay variation on each plate was between 4.81 and 7.94%, and the inter-assay variation was 6.68%.

Experiment 2: Effects of exogenous CORT, adrenal sensitivity (ACTH), and metyrapone on antipredator behavior

Seventy-seven animals were randomly assigned to four treatment groups. Each group was randomly divided into two different environmental chambers. Each animal was injected with 100 ul of the following mixed with amphibian ringer's solution (Carolina Biological Supply Company, Burlington, VA): 20pg CORT (Sigma Aldrich, Inc, St. Louis, MO, USA; n =17), 50pg ACTH (porcine, Sigma Aldrich, Inc, St. Louis, MO, USA; n = 20), 0.4mg Metyrapone (Sigma Aldrich, Inc, St. Louis, MO, USA) + 20 pg CORT (n = 20), or ringer's solution alone (n = 20). Injections were given by the same person and animals were then placed back into their enclosures for 60 minutes.

After 60 minutes, each animal was retrieved and stimulated to enter unken. The time each newt spent in unken was recorded. Thirty minutes after initial stimulation (90 minutes after the injection), we collected blood as described above. Blood was collected, processed, stored, and assayed as previously described. No skin biopsies were removed in the second set of animals used in Experiment 2.

Statistical Analyses

We analyzed the mass of the newts for each treatment group (for all experiments) to ensure that there were no statistical differences. For experiment 1A, we log-transformed CORT concentration and TTX concentration to meet the assumptions of normality. We analyzed both of these variables by treatment using a t-test. For experiment 1B, we regressed time spent in unken with both logCORT concentration and logTTX concentration. For experiment 2, we were unable to transform either CORT concentration or time in unken to meet assumptions of normality, so we used nonparametric measures. To compare time in unken and CORT concentrations among the treatment groups, we used a Kruskal-Wallis Test. Our *a priori* hypotheses stated that we anticipated differences between treatment groups (not just overall), thus post-hoc multiple comparisons were determined using the Wilcoxon Test. Further, we used Spearman rank correlations to determine the relationship between CORT and unken for each group. We were unable to compare newts between experiments 1 and 2 because individuals in experiment 2 were given injections, and time from initial stressor and time in captivity varied. We set our significance level at $\alpha = 0.05$. All statistics were performed using JMP 8.0.1 (SAS Institute Inc., 2009).

RESULTS

Experiment 1A

CORT concentration was significantly higher for newts in the stimulated treatment compared to the non-stimulated treatment group (t = 2.583, df = 11.412, p = 0.0124; Figure 2).

Experiment 1B

All experimental newts displayed the full unken reflex after stimulation (lifting head and tail > 90° above ground, Figure 1) and the mass of newts did not differ between treatment groups (t = 0.660, df = 16.71, p > 0.25). Tetrodotoxin levels did not differ between treatment groups (t = 0.243, df = 14.42, p = 0.81; Table 1), and there was no relationship between time in unken and TTX concentration (R^2 = 0.009, p > 0.5). Further, time in unken reflex was not correlated with CORT concentrations (R^2 = 0.02, p = 0.48, Figure 3).
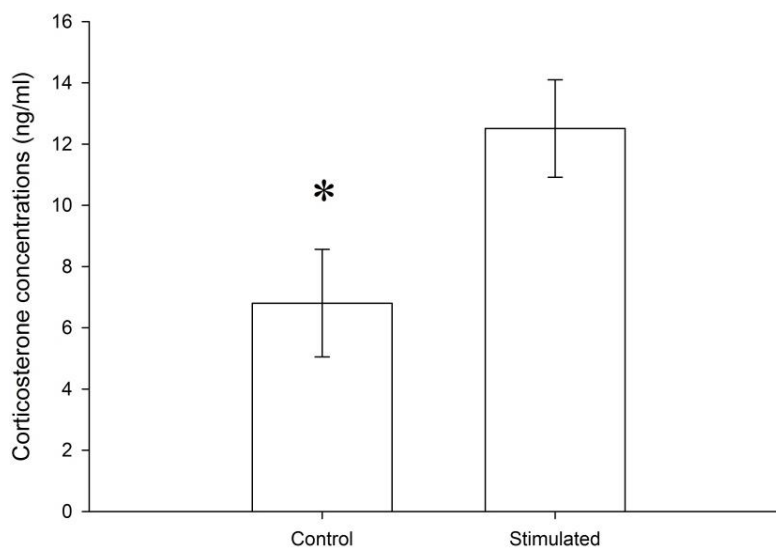
Fig. 4-2. *Experiment 1A*—Corticosterone levels in the stimulated group compared to the non-stimulated (control) group (t = 2.583, df = 11.412, p = 0.0124). Error bars indicate 1 ± standard error.

Table 4-1. *Experiment 1A and 1B*—Mass, corticosterone concentrations, tetrodotoxin concentrations, and time in unken reflex for *Taricha granulosa* (values are means ± SE).

Treatment	Mass (g)	Corticosterone (ng/ml)	Tetrodotoxin (ng/ml)	Time in unken (sec)
Control (n = 10)	13.21 ± 0.58	6.80 ± 1.76	8.72 ± 4.27	-
Stimulated (n=25)	13.67 ± 0.37	12.51 ± 1.59	8.36 ± 5.43	497 ± 93.76
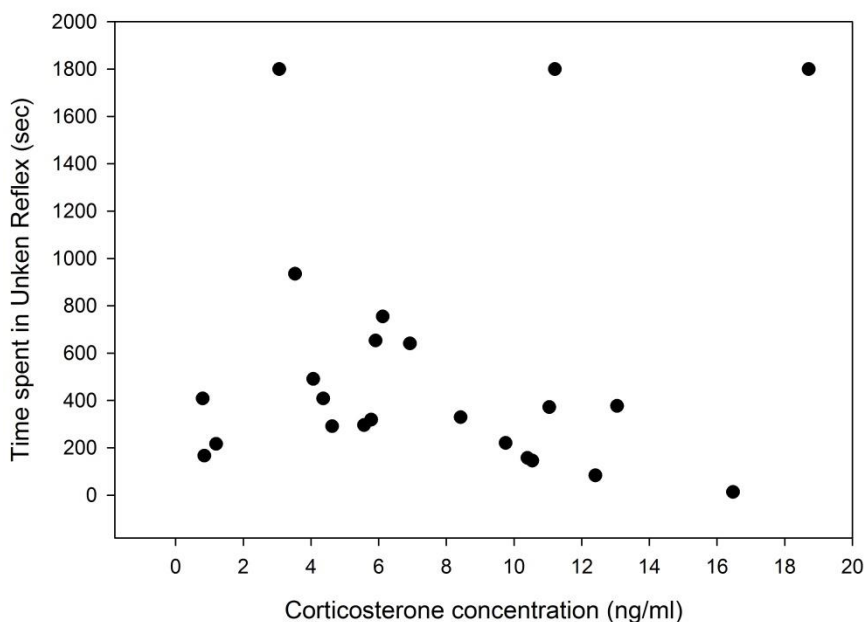
Fig. 4-3. *Experiment 1B*—Time spent in unken compared to corticosterone concentration 30 minutes after stimulation in Rough-skinned Newts ($R^2 = 0.02$, $p = 0.48$).

Experiment 2

  ACTH and CORT treatment groups had significantly higher levels of CORT than other treatment groups ($X = 56.12$, $df = 3$, $p < 0.0001$, Figure 4). Thus, metyrapone was effective at blocking CORT response. There was no overall difference in time spent in unken among the treatment groups ($X = 5.977$, $df = 3$, $p = 0.113$), but the CORT and ACTH treatment animals spent significantly less time in unken when compared to the metyrapone treated animals ($p < 0.05$, Figure 5). When time in unken was compared to CORT levels within each treatment group, CORT-injected animals demonstrated a

positive linear relationship ($r = 0.643$, $p = 0.05$), while there was no relationship between

the variables in the other three treatment groups ($r < 0.20$, $p > 0.4$; Figure 6).
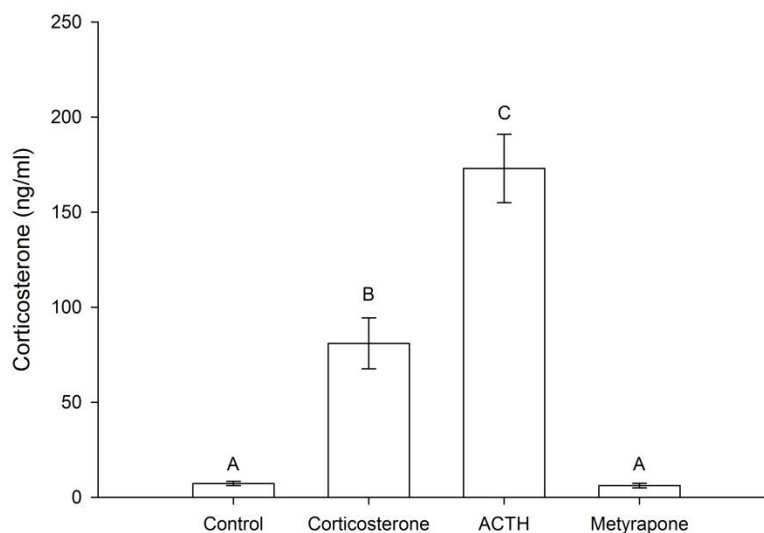
Fig. 4-4. *Experiment 2*—Corticosterone levels in four treatment groups (control, CORT, ACTH, metyrapone) 30 minutes after stimulation. Different letters indicate significance ($p < 0.05$). Error bars indicate $1 \pm$ standard error.
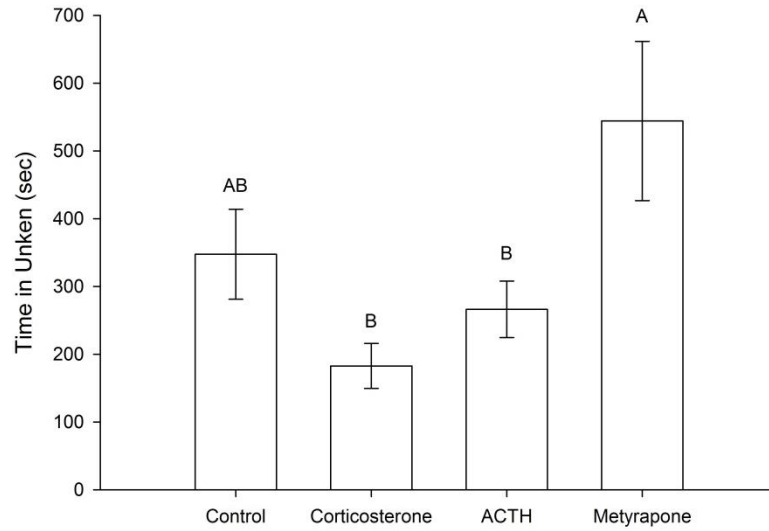
Fig. 4-5. *Experiment 2*—Time spent in unken in four treatment groups (control, CORT, ACTH, metyrapone) 30 minutes after stimulation. Different letters indicate significance ($p < 0.05$). Error bars indicate $1 \pm$ standard error.
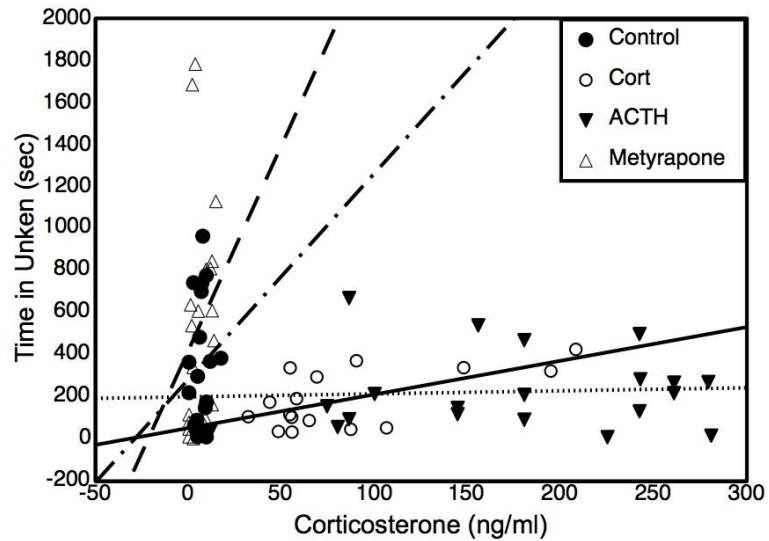
Fig. 4-6. *Experiment 2*—Corticosterone concentrations compared to time in unken for each of the four treatment groups (control, CORT, ACTH, metyrapone).

DISCUSSION

The results of our study indicate that exogenous, but not endogenous, CORT may have a suppressive effect on antipredator behavior in newts. However, we found no detectable relationship between the time in unken and levels of the defensive toxin TTX.

Our first study demonstrated the dramatic increase in circulating CORT levels between the control and stimulated animals, indicating that this simulated predator challenge represents an acute stress for these organisms. An acute increase in GCs in response to an unpredictable event, such as a predator attack, can be highly beneficial to an organism (Dhabhar, 2002; Dhabhar, 2009; Sapolsky et al., 2000). The increase in GCs can augment the immune system and shift energy resources from non-essential life processes (i.e., reproduction, digestion) to critical ones (i.e., increased cerebral blood flow, increased glucose metabolism; French et al., 2007; Sapolsky et al., 2000), including coping with any consequent wounds from the predator. Acute increases in GC levels also augment memory formation and have a myriad of effects on neuronal processes, which may be critical to avoiding subsequent predator attacks (de Kloet et al., 2008; Sapolsky et al., 2000; Thaker et al., 2010).

Endogenous levels of CORT did not predict time in unken, but in the treatment groups with CORT or ACTH applied, there was a suppressed antipredator response and thus with significantly higher levels of circulating corticosterone, unken time was significantly reduced. This observation may indicate that wild animals already exposed to

a stressful situation that have elevated CORT levels may not remain in unken for as long. Given the myriad of other potential dangers for these newts, suppression of this behavior may be beneficial. If, for example, an animal has been repeatedly attacked and has elevated CORT, it may be advantageous to abandon the unken posturing and physically escape a predator. Indeed, Lima and Bednekoff (1999) proposed that organisms may alter their antipredator behavior according to temporal changes in risk. Elevated CORT, in this case, may be a mediator of evaluating the risks between different antipredator strategies (escape or go into unken). It is important to note that levels of measured CORT in experiment 2 were higher than those in experiment 1 and in previously published work (Moore and Zoeller, 1985). These particular levels may not be ecologically relevant in ACTH or CORT animals. However, time in captivity, the injection, and increased time from first perception of stressor (injection) may have contributed to the increase, and no direct comparisons can be made.

While there is a relationship between these high exogenous CORT levels and time in unken, we cannot make conclusions as to how the two are linked. One puzzling finding is the high positive correlation between CORT levels and unken time in the exogenous CORT treatment group. We see an overall decrease in unken time in the exogenous CORT group when compared with the metyrapone and control groups, but the higher levels of CORT are closely associated with longer unken time. In this case, increased levels of CORT may increase the time that the newt stays in unken or the increased duration of the antipredator behavior may cause an increase in CORT concentrations. This complex relationship may be an example of a positive feedback loop, wherein the

increases in CORT levels from the stimulation create a more intense and prolonged response, which, in turn, increases the CORT levels. This positive feedback loop, also known as the "Challenge Hypothesis," has been well-studied and described in testosterone and male/male aggression during reproduction in birds (Wingfield et al., 1990). Interestingly, we did not see this relationship between the circulating CORT levels and unken in the ACTH treatment group. This may be due to the much higher levels of CORT in the ACTH group, which may be sufficient to trigger the negative feedback in the HPA axis (Sapolsky et al., 2000).

There are many factors which evidently alter the relationship between CORT and antipredator behavior, making it highly context-dependent. Individuals may physiologically respond differently depending upon seasons (Ricciardella et al., 2010), reproductive state (Hubbs et al., 2000), previous experience (Sandi et al., 1996), duration of exposure (Øverli et al., 2004), and type of stimulus (Bliley and Woodley, 2012). This context-dependence may be a result of the role of other components of the HPA response, such as changes in corticosteroid binding globulin, a plasma binding steroid (Breuner and Orchinik, 2002; Breuner et al., 2013), expression of membrane receptors (Moore and Orchinik, 1994; Orchinik et al., 1991), and/or the actions of other hormones (e.g. norepinephrine; Morilak et al., 2005).

Tetrodotoxin levels were not predictive of time spent in unken. However, the skin biopsies were taken prior to stimulation and so represent basal TTX concentrations. While there was no relationship between basal TTX levels and either of the other antipredator mechanisms, the amount of TTX released after stimulation is possibly

variable and should be examined. Therefore, there may be a relationship between TTX secretion and time spent in unken that was not detected. However, most work with honest signaling has been conducted with aposematic coloration and toxicity, not behavioral duration, and still no clear pattern has been established (Blount et al., 2009).

Our study presents preliminary evidence that CORT plays an important role in the antipredator behavior of the Rough-skinned Newt, although the direct mechanism linking the two is yet unclear. The relationship between antipredator behavior, toxicity, and glucocorticoids is evidently complex. Given that this behavior is critical to fitness, the relationship between the evolution of this behavior, toxicity, and endocrinological mechanisms should be further examined.

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CHAPTER 5

TO STRESS OR NOT TO STRESS: PHYSIOLOGICAL RESPONSES TO

TETRODOTOXIN IN RESISTANT GARTERSNAKES VARY BY SEX[4]

The activation of the hypothalamic pituitary adrenal (HPA) axis is one of the most

important physiological processes in coping with any deviation in an organism's

homeostasis. This activation and the secretion of glucocorticoids, such as corticosterone,

allow organisms to cope with perturbations and return to optimal physiological

functioning as quickly as possible. In this study, we examined the HPA axis activation in

Common gartersnakes (*Thamnophis sirtalis*) as a response to a natural toxin, tetrodotoxin

(TTX). This neurotoxin is found in high levels in the Rough-skinned Newt (*Taricha

granulosa*), which is a prey item for these snakes. To consume this toxic prey, these

snakes have evolved variable resistance. We hypothesized that the more resistant

individuals would show a lower HPA axis response than less resistant individuals, as

measured by corticosterone (CORT) and bactericidal ability, which is a functional

downstream measurement of CORT's activity. We determined "resistance level" for

tetrodotoxin from each individual snake by determining the dose which reduced race

speed by 50%. Individuals were injected then with an increasing amount of tetrodotoxin

(10, 25, and 50 MAMUs) to determine this value. Thirty minutes after every injection, we

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gathered blood samples from each snake. Our results show that, while there were no significant differences among individual CORT levels in a dose-dependent manner, female snakes did have a larger stress response when compared to both males and juveniles. Different life-histories could explain why females were able to mount a higher HPA axis response. However, TTX had no downstream effects on bactericidal ability, although juveniles had consistently lower values than adults. Our research shows a possible dichotomy between how each sex manages tetrodotoxin and gives way for a more comprehensive analysis of tetrodotoxin in an ecological context.

INTRODUCTION

Organisms have a suite of physiological responses to various external challenges, most of which are considered adaptive (Love and Williams, 2008; McEwen, 2007; Råberg et al., 1998). One of the most important and conserved of these responses is the initiation of the activation of hypothalamic-pituitary-adrenal axis (HPA; Wingfield and Romero, 2001; Wingfield, 2005). This system is responsible for interacting with other physiological systems by activating and/or suppressing functions, dependent upon context (Dhabhar, 2009; Sapolsky et al., 2000). While this activation has been examined in organisms for both anthropogenic and natural stressors, many questions remain as to the adaptive value of the HPA axis response and resulting physiological cascades (Dickens and Romero, 2013). There is a high amount of variation in both behavioral and neuroendocrine responses which are likely based on both genetic and environmental factors (Øverli et al., 2007; reviewed in Koolhaas et al., 1999; Korte et al., 2005).

Within this context, is the question of how organisms are adapted to respond to deviations from homeostasis that are regularly encountered within their specific environment. Theoretically, there should be an "optimal response," by which an organism does not over- or under-respond to these deviations (McEwen, 2007; Korte et al., 2005; Charmandari et al., 2004), especially if the challenge is frequently faced.

It would likely be beneficial for organisms to modulate their response based on evolutionary history with factors that might otherwise cause dramatic alterations in the homeostatic balance (Romero et al., 2009; Wingfield and Romero, 2001). Theoretical

examinations of the HPA axis provide evidence that its responses are likely influenced by evolutionary pressures (Ellis et al., 2006). Further, these examinations are supported by an ever-growing body of work that provides tangible examples. Variation exists between individuals, but also occurs between sexes and across life history stages (Lattin et al., 2012; Wada et al., 2008; Crespi et al., 2013; Moore and Jessop, 2003). These variances between sexes within species is especially important as the sexes often have disparate life history strategies due to the inherent cost of reproduction for females (Stearns, 1989; Madsen, 1987) and sex hormones which greatly influence the action of the HPA axis (von der Ohe and Servheen, 2002; Kajantie and Phillips, 2006; Wingfield and Sapolsky, 2003).

However, measuring HPA activation requires an examination of multiple factors (Breuner et al., 2013; Sapolsky et al., 2000). While glucocorticoids, such as corticosterone (CORT) in reptiles (Moore and Jessop, 2003), are most frequently used, these values have proven to be extremely complex (Dickens and Romero, 2013). Therefore, examining multiple measurements of HPA activation, such as downstream functional effects of CORT and the health of the animal, is necessary (Breuner et al., 2008). One increasingly common method is to analyze an organism's immune function (French et al., 2008). Acute increases in CORT typically correspond to increases in immune function, especially innate immunity (Dhabhar, 2002; Dhabhar and McEwen, 1997). However, studies in reptilian ecoimmunology have not shown consistent patterns as to the relationship between bactericidal ability and acute increases in CORT (Refsnider et al., 2015; Neuman-Lee and French, 2014; French et al., 2010), possibly because much

is unknown about the reptilian immune system (Zimmerman et al., 2010). Finally, examining the physical condition of animals is important when assessing HPA responses because this process is engaged to mobilize energy (Sapolsky et al., 2000). Energetic stores, as measured by body condition, can have a large impact on the resulting HPA activation (Jaatinen et al., 2013; Moore et al., 2005; Smith et al., 1994; Moore et al., 2000).

To examine the potential variation of the HPA response between individuals, sexes, and ontogenetic stages, a system with measured variation that could correspond to HPA activation and downstream effects should be tested. Gartersnakes (*Thamnophis sirtalis*) have adapted the ability to consume the highly toxic Rough-skinned Newt (*Taricha granulosa*), however, this trait is highly variable across populations and even within populations (Brodie and Brodie, 1990). For example, across populations, resistance varies from very high (>90 Mass-Adjusted Mouse Unit (MAMU); San Mateo, California) to very low (<4 MAMU; Bear Lake, UT) (Brodie and Brodie, 1990; Brodie et al., 2002). This is due to the fact that sympatric newt populations also have a high amount of variance in toxicity, which matches the snake resistance to toxicity (Hanifin et al., 1999). This pattern is evidence of an evolutionary arms race that results in increasing toxicity of the newts by the concurrent increase in toxicity resistance by the snakes (Brodie and Brodie, 1990; Brodie et al., 2002). However, there is variation in both the population of newts (Hanifin et al., 1999) and snakes (Brodie and Brodie, 1990) in toxicity and resistance, respectively. This system makes examining whether there is

variation in the HPA response among individuals and between sexes and ontogenetic stages in relation to exposure to the toxin.

Rough-skinned Newts secrete the potent neurotoxin tetrodotoxin (TTX) when threatened (Brodie, 1968). Tetrodotoxin binds competitively to sodium ion channels in muscle tissue and causes paralysis, which leads to suffocation and death (reviewed in Narahashi, 2001). Many populations of gartersnakes have evolved changes in the proteins of the sodium channel, which blocks the binding of TTX and therefore resists the effects (Geffeney et al., 2005). Because the muscle tissue is affected, resistance to TTX can be measured using the behavioral metric of sprint speed (Brodie and Brodie, 1990). Thus a snake's resistance can be measured by determining how fast it moves with varying concentrations of TTX relative to no TTX.

In this study, we wanted to determine the individual snake's resistance to TTX and relate this resistance to its stress and immune response. We tested reproductive-age male and females and juveniles to examine potential sex and ontogenetic differences in the response in a dose-response manner. We hypothesized that individuals with higher resistance would have a lower CORT response to TTX exposure, but that the response would be dose-dependent. We further hypothesized that innate immunity would be positively correlated with increasing levels of CORT. Finally, we predicted that these relationships would be consistent across both sex and ontogenetic stage.

METHODS

Collection

Common gartersnakes (*Thamnophis sirtalis*) were hand collected in April 2014 in Benton County, OR (n = 41; Females = 21, Males = 12, Juveniles = 8), and transported in individual opaque bags to Utah State University within 10 days of capture. During these 10 days, snakes were kept in opaque bags and allowed access to water for 1 hour each day. Each individual was housed separately in a glass aquaria (37.8L) with newspaper substrate, water dish, and plastic hide box with moist sphagnum moss. Air temperatures were maintained at 27°C, snakes were allowed a thermal gradient using heat tape, and light was on a 12:12 on:off cycle. Snakes were all offered one meal of thawed mice (approximately 10% of their body mass) six days after arriving in the laboratory. All uneaten food was removed and recorded. Fifteen days after arrival at the laboratory, each snake was weighed to the nearest tenth of a gram, the snout-vent length was determined (SVL, distance from snout to cloaca), and the number of follicles in females was counted using an ultrasound (Sonosite, Micromaxx). All procedures were approved by the Utah State University IACUC (Protocol #2299).

Racing and determining resistance level

This bioassay was conducted as described by Brodie and Brodie (1990). Two days after measurements, we began the testing. All animals were tested on the same day in the same manner so that animals had been housed in captivity for the same amount of

time. Briefly, twenty-four hours prior to racing, all snakes were removed from heat tape

to ensure that each individual was the same temperature, as temperature affects racing

speed (Brodie and Russell, 1999). To test maximal racing speed, each individual was

removed from the aquaria and placed on a three-meter racetrack lined with artificial turf.

The investigator then lightly taps the tail of the snake to simulate a predator to ensure that

the snake moves as quickly as possible. Four half-meter sections are measured using a

digital timer and the fastest time is used. For the baseline measurement, this process was

repeated twice, four hours apart, and the speeds averaged. All future racing speeds were

calculated as a percentage of this maximal speed. Previous work has shown that snake

speed is slowed linearly with TTX injections (Brodie and Brodie, 1990; Brodie et al.,

2002). Therefore, by determining the dose at which the speed of the snake is reduced by

50% of the control run, individual resistance level can be calculated. Hereafter this is

referred to as "resistance level."

Dosing and collecting blood samples

The day after the baseline race, each snake was given an intra-coelomic injection

with a mass-adjusted dose of Ringer's solution. The snake was promptly put back into its

aquarium for exactly 30 min. At 30 min, the snake was removed and raced down the

track as described above. A blood sample was then collected via the caudal vein within

one minute. All samples were stored on ice for less than one hr and centrifuged at 2400

rpms. The plasma was separated from the red blood cells and stored at -80°C until further

processing.

Two days later, the snakes were injected with 10 mass-adjusted mouse units

(MAMUs, i.e. the amount of TTX required to kill one gram of mouse; (Brodie and

Brodie, 1990). This inital dose was selected due known resistance from previous

investigations of this population. After 30 minutes, snakes were raced and bled as

described before. The same process was repeated two days later using 25 MAMUs and

then two more days later using 50 MAMUs. The maximum time that this took an

individual was eight days. Because of the variation within the population, if an individual

snake proved to be less resistant than 50% at any of the doses (its speed was decreased

more than 50% at a given dose), it was not given the next highest dose to prevent killing

it. In these cases, the snake's blood was not used in the statistical means given that it did

not receive the same dose. The following are number of animals tested at Baseline:

Females (F) = 21, Males (M) = 12, Juveniles (J) = 8; 10 MAMUs: F = 21, M = 12, J = 8;

25 MAMUs: F = 17, M = 11, J = 4; 50 MAMUs: F = 11, M = 8, J = 4.

Radioimmunoassay

Circulating corticosterone levels were determined using a previously described

protocol (French et al., 2010; French et al., 2006; Moore, 1986). Samples were extracted

using isooctane: ethyl acetate, dried, and resuspended in PBS buffer. Samples were

assayed in duplicate for CORT (MP Biomedicals, Lot #3R3PB-19E) and the mean of the

two were used in analysis. For each sample, we used an aliquot of the resuspended

fractions to measure individual recoveries following extraction and chromatography.

These recoveries were used to adjust final sample concentration values to account for any

losses during these procedures. Standards of known value and negative controls were included in every assay as a reference to ensure accuracy. All samples were run in a single assay. Intra-assay variation was 12.1% and accuracy was 103.6%.

Bactericidal Ability

We performed the bactericidal assay (BKA) to measure innate immune function, following the protocol outlined in French and Neuman-Lee (2012). Briefly, we combined a 1:4 dilution of plasma with CO_2-Independent media (Gibco, Grand Island, NY) plus 4nM L-glutamine (Sigma-Aldrich), and 10^5 CPU (colony producing unit) *Escherichia coli* (EPowerTM Microorganisms #0483E7, ATCC 8739, MicroBioLogics, St. Cloud, MN), and agar broth on a 96-well microplate. We calculated the background absorbance using BioRad xMark microplate reader. After a 12-hour incubation, we again read the absorbance and calculated the bactericidal ability by dividing the mean absorbance for each sample (run in duplicate) by mean absorbance for the positive controls (containing only media and bacterial solution), and multiplying by 100. This provides the percent bacteria killed relative the positive controls. Negative controls (containing media only) were also run to ensure contamination was absent. Inter-assay variation between plates was 1.1%.

Statistical Analyses

Body condition was calculated by taking the residual of mass and SVL (Neuman-Lee et al., 2015b) for each individual. Linear regressions of the residual against

physiological measures were calculated for all snakes and within each sex class. Further,

the number of follicles was regressed against the residuals for females.

Corticosterone was not normally distributed, so we $\log_{10}$-transformed the data for

all analyses. We first measured possible differences between the sexes and juveniles

(Male, female, juvenile) at each time-point (increasing values of TTX) using an analysis

of variance (ANOVA). When appropriate, we compared the groups using a Tukey's

pairwise comparison. To measure the effect of time on the sexes, we conducted a

repeated measures ANOVA (rmANOVA). Individuals which had missing data were

dropped from the rmANOVA. We repeated this procedure for the bactericidal ability,

although the data were not normal and no transformations were successful. Therefore, we

conducted a Wilcoxon/Kruskal-Wallis to determine sex effects and a Wilcoxon each pair

test for pair-wise comparisons.

We measured the possible differences in resistance level between the sexes and

juveniles by $\log_{10}$-transforming the resistance values and conducting an ANOVA. To

determine the possible effect of individual resistance level on corticosterone and

bactericidal ability, we conducted linear regressions of the individual resistance level

against the $\log_{10}$ CORT. We could not meet the assumption of normality for the

bactericidal ability data, thus we conducted a Spearman's test. Bonferroni corrections

were made to all linear regressions to reduce a Type I error and for these tests, the $\alpha =$

0.003.

Females were separated by those with verifiable follicles against those that were

non-reproductive. A t-test was conducted then conducted for CORT and resistance level,

a Wilcoxon test for bactericidal ability. All analyses were conducted in JMP 11.0 (SAS Institute, Inc, 2013).

RESULTS

Body Condition

Mass was significantly different between sex classes ($F_{(2,38)} = 80.97$, $p < 0.0001$), with females being the largest and juveniles being the smallest (g $\pm$ 1 standard error; Females 51.79 $\pm$ 5.38, n = 21; Males 31.32 $\pm$ 2.33, n = 12; Juveniles 6.95 $\pm$ 0.49, n = 8).

Body condition was significantly different among the three groups (Male, female, juvenile; $F_{(2,38)} = 18.84$, $p < 0.0001$), with no significant difference between the female and juveniles, but a difference between both of them and males. However, this was likely due to females having a higher condition due to developing follicles and thus made the females have larger mass. The number of follicles each female had was strongly positively related with female body condition ($R^2 = 0.72$, $p < 0.0001$).

Body condition had varying relationships with corticosterone. Overall, body condition did not affect corticosterone concentrations at any time point ($p > 0.09$). When each sex was analyzed, body condition was negatively correlated with CORT only for males at 25 MAMU (Table 1, $R^2 = 0.68$, $F_{(1,10)} = 21.32$, $p = 0.001$). Body condition was not related to bactericidal ability at any time point ($p > 0.1$).

Table 5-1

Body condition (residual of snout-vent length and mass) vs CORT concentrations (log-transformed) in gartersnakes (*Thamnophis sirtalis*) after increasing doses of TTX. Asterisk indicates significance after Bonferroni correction ($\alpha = 0.003$).

	Baseline	10 MAMU TTX	25 MAMU TTX	50 MAMU TTX
All snakes	$R^2 = 0.002$ $F_{(1,39)} = 0.08$ $p = 0.78$	$R^2 = 0.05$ $F_{(1,39)} = 2.15$ $p = 0.15$	$R^2 = 0.07$ $F_{(1,38)} = 2.98$ $p = 0.09$	$R^2 = 0.13$ $F_{(1,20)} = 2.96$ $p = 0.10$
Male snakes	$R^2 = 0.22$ $F_{(1,10)} = 2.85$ $p = 0.12$	$R^2 = 0.35$ $F_{(1,10)} = 5.42$ $p = 0.042$	$R^2 = 0.68$ $F_{(1,10)} = 21.32$ $p = 0.001*$	$R^2 = 0.51$ $F_{(1,5)} = 5.17$ $p = 0.07$
Female snakes	$R^2 = 0.04$ $F_{(1,19)} = 0.84$ $p = 0.37$	$R^2 = 0.002$ $F_{(1,19)} = 0.05$ $p = 0.83$	$R^2 = 0.10$ $F_{(1,18)} = 2.07$ $p = 0.17$	$R^2 = 0.37$ $F_{(1,9)} = 5.38$ $p = 0.046$
Juvenile snakes	$R^2 = 0.50$ $F_{(1,6)} = 5.98$ $p = 0.05$	$R^2 = 0.01$ $F_{(1,6)} = 0.08$ $p = 0.79$	$R^2 = 0.15$ $F_{(1,2)} = 1.09$ $p = 0.34$	$R^2 = 0.57$ $F_{(1,2)} = 2.66$ $p = 0.244$

Reproduction

At the onset of the study, 14 of the 21 females had verifiable follicles. No factors (CORT, BKA, resistance level) were influenced by follicle presence ($p > 0.15$).

Corticosterone

At baseline (after saline injection), there was no difference in CORT concentration between the sexes (Male, Female, Juvenile; Figure 1, $F_{(2,38)} = 0.921$, $p = 0.41$). The rmANOVA revealed a significant effect of sex and sex*time (Time: $F_{(3,95.8)} = 1.01$, $p = 0.39$; Sex: $F_{(2, 38.3)} = 12.62$, $p < 0.0001$; Sex*Time: $F_{(6, 95.7)} = 4.86$, $p = 0.0002$). After the first toxin injection (10 MAMU), female levels were significantly higher than either juveniles or males ($F_{(2,38)} = 11.84$, $p < 0.0001$). Males and juveniles did not significantly differ from each other. Female levels of CORT remained significantly

higher for both the second ($F_{(2,37)} = 14.76$, $p < 0.0001$) and third ($F_{(2,20)} = 4.80$, $p = 0.02$) toxin injections.

Bactericidal ability

At baseline, there was a significant difference between the juveniles and either males or females, but not between males and females (Figure 2, $X^2 = 8.55$, $p = 0.014$, d.f. 2). Juvenile bactericidal ability continued to be significantly lower than either males or females for the remaining injections (1st: $X^2 = 9.89$, $p = 0.007$; 2nd: $X^2 = 12.96$, $p = 0.002$; 3rd: $X^2 = 8.55$, $p = 0.014$).
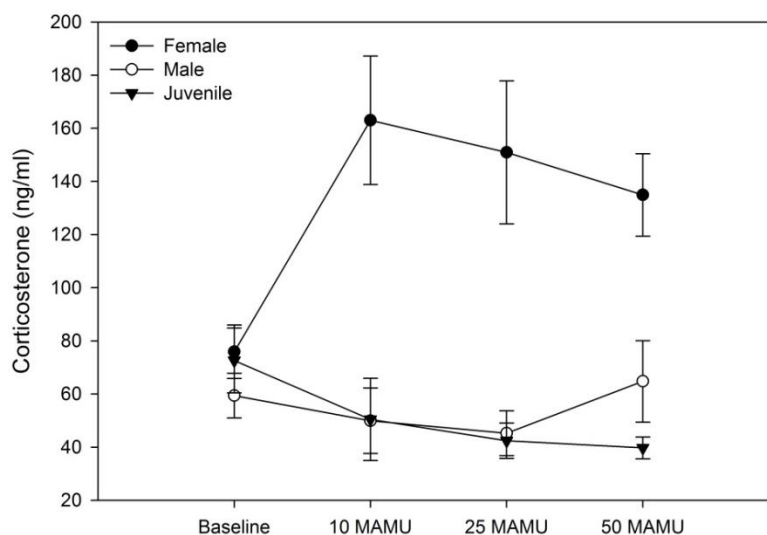
Fig. 5-1. Corticosterone levels (ng/ml) in female, male, and juvenile gartersnakes (*Thamnophis sirtalis*) 30 min after exposure to increasing levels of TTX and immediately after racing. Error bars indicate ± 1 standard error. Sample sizes, in the following order: BL, 10 MAMU, 25 MAMU, and 50 MAMU, for females (21, 21, 17, 11), males (12, 12, 11, 7), and juveniles (8, 8, 4, 4).
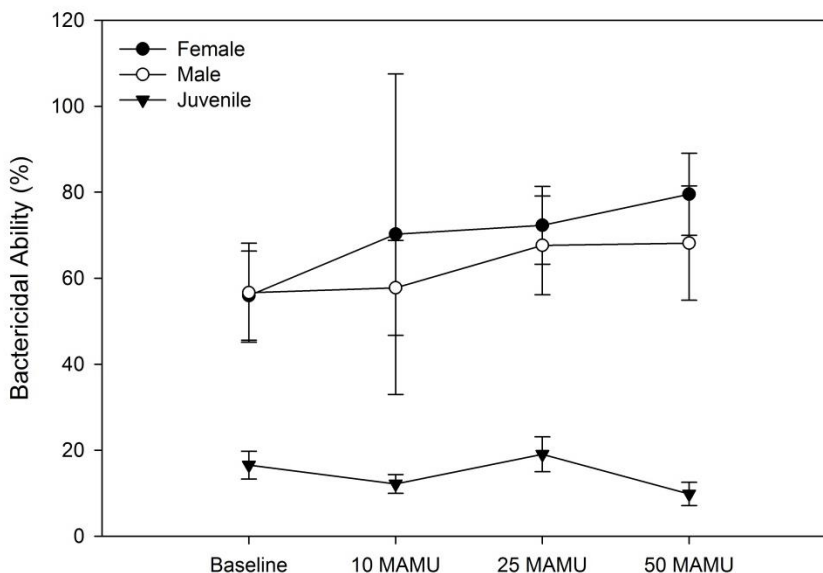
Fig. 5-2. Bactericidal ability in female, male, and juvenile gartersnakes (*Thamnophis sirtalis*) 30 min after exposure to increasing levels of TTX and immediately after racing. Error bars indicate ± 1 standard error. Sample sizes, in the following order: BL, 10 MAMU, 25 MAMU, and 50 MAMU, for females (16, 16, 14, 10), males (12, 12, 11, 8), and juveniles (8, 5, 4, 4).

The rmANOVA reflected these analyses showing no significant effect of time, but only of sex (Time: $F_{(3,85.4)} = 0.87$, $p = 0.46$; Sex: $F_{(2, 38.7)} = 11.39$, $p < 0.0001$; Sex*Time: $F_{(6, 85.1)} = 0.44$, $p = 0.85$).

Resistance

There was no difference among the sexes (Male, Female, Juvenile) in terms of resistance level ($F_{(2,38)} = 0.832$, $p = 0.44$; Resistance in MAMUs ± 1 standard error: Females $42.67 ± 10.80$, Males $48.76 ± 8.82$, Juveniles $45.86 ± 17.66$). There was a high amount of variation between individuals (Figure 3).
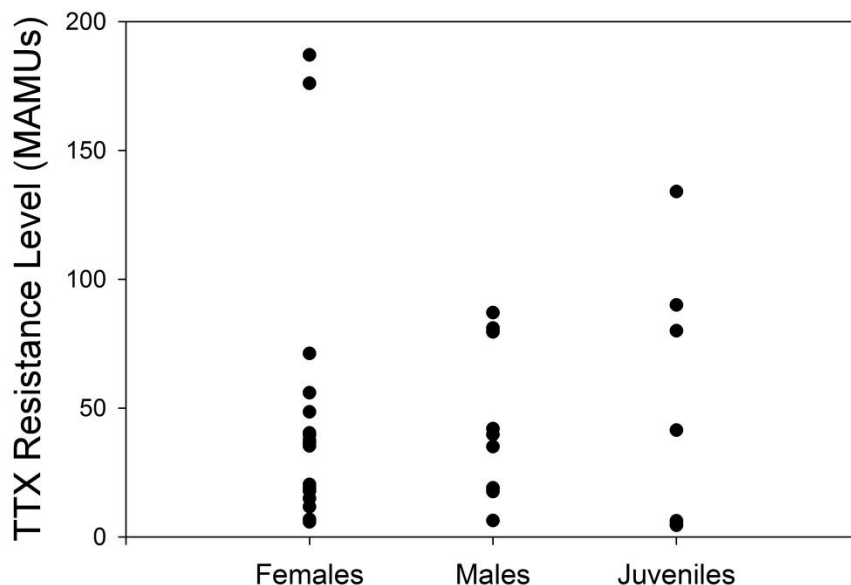
Fig. 5-3. Resistance levels in individual gartersnakes (*Thamnophis sirtalis*) to tetrodotoxin (TTX) as calculated by the concentration of TTX which decreased their race speed by 50%. Units are given as mass-adjusted mouse units (MAMU), which is a standardized amount of TTX used in previous studies (see Brodie and Brodie, 1990).

There was no relationship between individual resistance level and CORT or bactericidal ability, regardless of time-point (p values between 0.17 and 0.98). Further, none of the parameters were related to individual resistance level (p values between 0.13 and 0.91).

DISCUSSION

The current study provides evidence that female gartersnakes may respond differently to TTX than either males or juveniles. Females had an elevated CORT response after the first TTX injection that remained throughout the next three injections,

while the males and juveniles did not elevate their CORT levels above baseline at any dose. Individual TTX resistance was not related to CORT levels. This CORT response was not related to body condition in females, yet was negatively correlated to body condition for males at the second (25 MAMU) injection. Finally, TTX and CORT had no apparent effect on bactericidal ability, although overall juveniles had significantly lower innate immune function than adults.

Contrary to our predictions, our study showed no evidence that individual snakes modulated their CORT response to TTX based on their individual resistance, as a measure of homeostatic deviation. However, evidence that fitness and health of an organism is directly related to CORT has proven elusive (Bonier et al., 2009a; Bonier et al., 2009b; Dickens and Romero, 2013) and additional measurements, such as other immune metrics and corticosteroid binding globulins, may be necessary to provide a clearer picture of the mechanisms behind the HPA axis functioning (Breuner et al., 2013).

When examining the additional measure of immune function, we found no effect of TTX in either sex. Juveniles had a consistently lower level of bactericidal ability, as is seen in many species where juveniles do not have a fully developed immune system. Bactericidal ability can be seen as a functional measurement of health given that organisms in the wild must frequently cope with pathogens (French and Neuman-Lee, 2012). In reptiles, bactericidal ability is especially useful given the high reliance on non-specific innate immune responses (Zimmerman et al., 2010).

The increase in CORT associated with TTX exposure for females, but not males or juveniles, may occur for many reasons, none of which are mutually exclusive: 1) It

may be adaptive for females to have a stronger stress response, 2) Males may be

energetically less able to mount a stress response after emergence and mating, 3) Females

may be able to redirect energy from developing follicles or existing fat stores toward the

stress response, 4) TTX may serve as an anticipatory signal for larger snakes (females)

that a meal is about to be ingested, and/or 5) Other physiological mechanisms, such as

sex steroids, play a role in enhancing the stress response.

A key component of most life-history trade-off theories is that a high amount of

energetic investment is required to reproduce (Stearns, 1989; Wade and Schneider, 1992).

However, in the vast majority of organisms, the female expends the bulk of this energy

by producing eggs, carrying eggs/embryos, supplying nutrients to developing embryos,

and/or providing nutrients after parturition (lactation (Wade and Schneider, 1992;

Blackburn and Flemming, 2009)). Therefore, it is likely that there are major differences

by which males and females have adapted due to this inherent dissimilarity in energetic

expenditure for reproduction. It may be beneficial for sexes to have a different reaction

norm to a similar stressor. Females also may have a higher stress reactivity because they

must have a more proactive coping style to ensure not only her survival, but that of her

young (Kajantie and Phillips, 2006). However, in some species, females show a reduced

stress response relative to males to prevent abandoning already vested eggs or offspring

(Bokony et al., 2009). Females in this study, however, were still early in reproduction and

therefore able to resorb follicles if necessary.

The second possibility for the lack of response in the males is that males had a

negative energy balance and were therefore unable to mount a stress-response

(Wingfield, 2005). Moore et al. (2000) provided evidence that males from this population have low body condition after emerging from overwintering, likely due to the lack of prey. Interestingly, this study found that males have the some of the lowest concentrations of CORT during this time frame as well. This study did not examine females, but another study with gartersnakes showed that females have a significantly higher fat body index than males throughout and after hibernation (Costanzo, 1985).

In conjunction with emerging from hibernation with a lower body condition, males also typically emerge earlier than females (Gregory, 1974; Shine et al., 2001), forcing them to use more energetic stores while waiting for females to emerge. Males may expend this valuable energy in courtship and vying for a female and will often remain at the hibernaculum site for a longer period of time, while females will disperse rapidly and likely begin foraging (Gregory, 1974; Shine et al., 2001). While gartersnakes do not display energetically costly combat as in other species (e.g. rattlesnakes), they still must compete to mate with the female (Shine and Mason, 2005). Throughout the entire mating period, male gartersnakes undergo a period of anorexia, further compounding their lower body condition (O'Donnell et al., 2004).

Thirdly, in capital breeders, such as snakes, the majority of the energetic requirements for reproduction is obtained prior to or early in reproduction (Doughty and Shine, 1997), allowing females the possibility to divert this energy away from developing follicles.

Our fourth explanation of the enhanced female reactivity comes from the nature of the source of TTX. Rough-skinned newts average 5.7-8.9 cm (Stebbins, 2003), and

therefore only larger snakes may be regularly ingesting adult newts. There is a considerable amount of work which shows that CORT increases in individuals as an anticipatory hormone prior to eating (Stephan, 2002; Krieger, 1974; Honma et al., 1984). While this model has mostly been applied to lab rodents that are on a regular feeding schedule, the mechanism behind this repeated observation has not been fully determined (Stephan, 2002). When a snake first bites a newt, the newt begins secreting TTX, thus exposing the snake orally to the toxin. This may serve as a signal that food will be ingested shortly. However, this seems an unlikely scenario given that there is a high latency to eating in snakes (Rossman et al., 1996) and an inherent unpredictability for predators to find prey in the wild.

The fifth possibility states that other physiological mechanisms inherent in females may interact with and enhance CORT concentrations. A large body of research has revealed that females will often have increased levels of glucocorticoids after experiencing a stressor, likely due to elevated estradiol levels (reviewed in Kudielka and Kirschbaum, 2005). Estradiol is known to enhance corticotropin-releasing factor gene transcription in humans (Vamvakopoulos and Chrousos, 1993), increase both adrenocorticotropic hormone and glucocorticoids (Kitay, 1963), decrease receptor-mediated negative feedback (thereby increasing CORT responsiveness (Burgess and Handa, 1992)), and increases the production of corticosteroid-binding globulins (Moore et al., 1978). Taken together, it is possible that the stress-response to TTX was, in part, enhanced by estradiol.

Given the design of the study, whereby we controlled for the effects of injection and antipredator behavior in order to isolate the effects of TTX on CORT, we were unable to take true baseline CORT measurements. However, males and juveniles did not mount an additional response to TTX when compared to vehicle control, whereas females did, providing strong evidence for sex differences in reactivity. Interestingly, we found a different result when we subjected a subset of adult individuals in this study to an acute handling stressor (baseline sample then 30 minutes after initial capture) in the field. From these data, we know that the sexes responded similarly to this acute handling stress (Males (n = 12) baseline CORT = 21.8 ± 6.8 and stress-induced CORT = 88.6 ± 18.9 and Females (n = 13) baseline CORT = 19.4 ± 6.8 and stress-induced CORT = 93.9 ± 18.9 Neuman-Lee, unpublished data). Moreover, in a different study testing the effects of an anthropogenic contaminant (polybrominated diphenyl ether), female snakes did not have elevated CORT throughout the study nor a significant change in magnitude compared to the control (Neuman-Lee et al., 2015a). An additional complication in our interpretation was the increasing dose of TTX with each snake. We may have seen different results had we tested snakes with only a higher dose instead of starting at a lower dose and then increasing the dosage.

We used behavior as a metric of resistance (standardized protocol in TTX research) and thus our study also tested possible relationships between antipredator response and CORT. While some studies have shown certain antipredator behaviors are associated with elevated levels of CORT (Thaker et al., 2009a, b), CORT may not always be correlated with behavior (Neuman-Lee et al., 2015). In this case, it is likely that the

physiological effects of TTX (i.e. paralysis of muscular tissue (Narahashi, 2001)) are the primary control mechanisms of racing antipredator behavior.

Our current study demonstrates a clear difference between sexes in responses to an ecologically and evolutionarily relevant toxin. This is surprising given that both sexes are known to ingest toxic newts in the wild, and that the sexes respond similarly to other controlled stressors (capture and handling stress). The difference between the sexes in the hormonal response to tetrodotoxin illustrates a potential divergence in strategies for coping with a toxic byproduct of a valuable food source. This divergence may be driven by classic evolutionary reproductive conflicts in energy use and requirements or physiological differences between the sexes. However, we did not see divergence in bactericidal ability between the sexes, indicating that TTX exposure may not be related to innate immune function. More work is needed testing responses to TTX across varying reproductive and energy states to disentangle the sex related differences that we observed.

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CHAPTER 6

COMPARING THE NATURAL AND ANTHROPOGENIC SODIUM

CHANNEL BLOCKERS TETRODOTOXIN AND INDOXACARB IN

GARTERSNAKES[5]

Synthetic chemicals, such as pesticides, are used in a variety of ways in the

agricultural industry. Anthropogenic chemicals pose a unique challenge to organisms

because of the lack of evolutionary history between the chemical and the organism.

However, research has shown that some organisms have a resistance to these synthetic

chemicals due to their evolved resistance to a natural compound with a similar structure

or mode of action. Indoxacarb (INDOX) is a relatively new pesticide with a similar mode

of action to that of tetrodotoxin (TTX). Tetrodotoxin is a naturally occurring toxin that is

used as an antipredator defense in the Rough-Skinned Newt (*Taricha granulosa).* Some

populations of the Common Gartersnake (*Thamnophis sirtalis)* have developed a

resistance to tetrodotoxin. Here, we investigated the correlation between TTX and

INDOX resistance in snakes. We hypothesized that INDOX would induce a much higher

stress response than the naturally occurring TTX. We injected each snake with

tetrodotoxin (1 MAMUs). We did the same with mass-adjusted units of INDOX. We

measured corticosterone, testosterone, and bactericidal ability. Our results show an acute

stress response to INDOX, but not TTX through an elevate corticosterone and innate

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[5] Coauthored by Lorin A. Neuman-Lee, Edmund D. Brodie, Jr., Tyler Hansen, Edmund D. Brodie III, and Susannah S. French SS. Reprinted with permission from Journal of Experimental Zoology In press.

immune response, although there was no difference in testosterone concentration. These results suggest that, although INDOX may have a similar mechanism of action, gartersnakes do not react in a similar manner as to TTX. This research gives a physiological perspective on the differences between naturally occurring compounds and synthetic compounds.

INTRODUCTION

A wide variety of chemicals are permeating the environment at an unprecedented rate, which poses unique challenges to organisms. Synthetic chemicals are particularly problematic because organisms lack an evolutionary history with them. In numerous cases, organisms have evolved resistance to naturally occurring chemicals, even when these same chemicals are highly toxic to other organisms (Brodie and Brodie, '90; Hutchinson et al, '07; Mebs, '98). If an organism has developed an evolutionary resistance to a natural toxin, it may provide a small reprieve whereby the organism has inadvertently evolved resistance to a synergistic chemical with a similar structure and/or a similar mode of action. This has been found in many species of insects which have proven to be resistant to both natural plant chemical defenses as well as pesticides with a similar mechanism of action (reviewed in Després et al, '07).

One highly toxic natural chemical is tetrodotoxin (TTX). This defensive mechanism is found across the phylogenetic tree, from marine animals such as the puffer fish, to the western newts *Taricha granulosa*, and even some terrestrial invertebrates (Stokes et al, '14). Structurally, TTX is a guanidine compound attached to an oxygenated carbon skeleton (Chau et al, '11). TTX's mechanism of action is attaching itself to TTX-sensitive voltage-gated sodium channels, inhibiting them from carrying out action potentials in excitable nerve and muscle tissue. This results in paralysis of any tissue controlled by TTX-sensitive sodium channels. In mammals, it paralyzes the diaphragm (Brodie, '68).

This natural toxin is also unique because of the well-documented role it plays in a co-evolutionary arms race between two North American vertebrates, the Rough-skinned Newt (*Taricha granulosa*) and the Common Gartersnake (*Thamnophis sirtalis*). Newts are able to secrete TTX from granular glands on their dorsal surface as a defense mechanism (Brodie, '68). Through genetic mutation of the sodium ion channel, sympatric gartersnakes have evolved resistance to TTX and are effective predators of newts (Brodie and Brodie, '90; Brodie et al, '02; Feldman et al, '09). While the level of toxin in newts and resistance in snakes varies across the species' ranges, in general populations of highly toxic newts are sympatric with populations of highly resistant snakes (Brodie et al, '02; Hanifin et al, '99).

With a similar mode of action to TTX, the relatively new pesticide indoxacarb (INDOX) is used to control lepidopteran pests on fruits and vegetables (McCann et al, '01). While little is known about the effects of this oxadiazine pesticide on vertebrates, research indicates that the mode of action is similar. In a study performed on rats, the effects indoxacarb on TTX-sensitive and TTX-resistant sodium channels were examined. This study found an irreversible inhibition in the action potential in both channels, but the magnitude was approximately twice as great in the TTX-sensitive channels (Zhao et al, '03). Hematological, immunological, and behavioral effects of indoxacarb were also tested using mice and rats (Shit et al, '08).

Given that virtually all organisms have a finite amount of energy that must be distributed to various functions, exposure to any form a stress can cause an imbalance in energy allocation (Wingfield, '05; Wingfield and Romero, '01). Organisms that are

adapted to their environment should have an optimal reaction to normal perturbations within that environment, even chemical exposures (Romero et al, '09). Therefore, testing the perceived magnitude of stress on an organism and the downstream effects can elucidate how well an individual reacts to changes in its environment (Angelier and Wingfield, '13; Walker et al, '05). One of the potential mediators of the energetic shifts during a stressful event are glucocorticoids (GCs). These are energy-mobilizing hormones and can be energetically costly to secrete, although it is often necessary to do so in stressful situations (Wingfield and Romero, '01). Organisms must also allocate their limited energy to self-maintenance processes such as immune function and tissue and cellular repair (French et al, '06; French et al, '07). Glucocorticoids have also been well documented on their effects on reproductive ability. Specifically, glucocorticoids suppress the hypothalamic-pituitary-gonadal axis and subsequently the release of gonadotropin-releasing hormone, which results in lower reproductive function (Whirledge and Cidlowski, '10).

To examine whether gartersnakes have a similar response to the natural toxin with which they have evolved (TTX) compared to an anthropogenic toxin with the same mechanism of action (INDOX), we designed two experiments. In the first experiment, we tested whether snakes respond to increasing doses of TTX absent from any other potential stressor (i.e., racing). In the second experiment, we gave a different set of snakes both TTX and INDOX to measure response. We predicted that snakes would have minimal to no response to the increasing doses of TTX. Further, we hypothesized that exposure to the synthetic toxin INDOX would elicit a much higher stress response in

snakes than the naturally occurring TTX to which they have evolved a resistance. This higher stress response would necessarily require trade-offs in energetic allocation, with likely a decrease in self-maintenance energy and/or reproductive investment.

METHODS

Collection

Male gartersnakes (*Thamnophis sirtalis*) were hand-collected in Cache County, UT in 2014 as they emerged from their hibernacula. Each individual was transported immediately to Utah State University and housed separately in glass aquaria (37.8L) with newspaper substrate, water dish, and plastic hide box with moist sphagnum moss. Air temperatures were maintained at 27°C, snakes were allowed a thermal gradient using heat tape on a 12L:12D cycle. Each snake was weighed to the nearest tenth of a gram and the snout-vent length was determined (SVL, distance from snout to cloaca). All procedures were approved by the Utah State University IACUC (Protocol #2299).

Experiment 1: Dose-Response to TTX

Twenty snakes were injected in the intra-coelomic cavity with a mass-adjusted dose of Ringer's solution. After 30 minutes, a blood sample was obtained via the caudal vein. This 30 minutes was selected because it is the time at which TTX has the maximal effect on snake motor function (Brodie and Brodie, '90; Brodie et al, '02). Snakes fully recover from TTX injections after 24 hours (Brodie and Brodie, '90). After 48 hours,

each snake was injected with 1 mass-adjusted mouse unit (MAMUs, i.e. the amount of

TTX required to kill one gram of mouse; (Brodie and Brodie, '90) and a blood sample

was taken after 30 minutes. After another 48 hours, snakes were injected with 3 MAMUs

and bled after 30 minutes. Finally, the last injection of 5 MAMUs was given after 48

hours and snakes were bled after 30 minutes. All samples were stored on ice for less than

one hr and centrifuged at 2400 rpms. The plasma was separated from the red blood cells

and stored at -80°C until further processing.

Experiment 2: TTX versus INDOX responses

<u>*Racing and determining 50% resistance*</u>

This bioassay was conducted as described by Brodie and Brodie ('90). Briefly,

twenty-four hours prior to racing, all snakes were removed from heat tape to ensure that

each individual was the same temperature, as temperature affects racing speed (Brodie

and Russell, '99). To test maximal racing speed, each individual was removed from the

aquaria and placed on a three-meter racetrack lined with artificial turf. The investigator

then lightly taps the tail of the snake to simulate a predator to ensure that the snake moves

as quickly as possible. Four half-meter sections are measured using a digital timer and the

fastest time is used. For the baseline measurement, this process was repeated twice, four

hours apart, and the speeds averaged. All future racing speeds were calculated as a

percentage of this maximal speed. Previous work has shown that snake speed is slowed

linearly with TTX injections (Brodie and Brodie, '90; Brodie et al, '02). Therefore, by

determining the dose at which the speed of the snake is reduced by 50% of the baseline, individual 50% resistance can be calculated.

Dosing with TTX and INDOX

The day after the baseline race, each snake was given an intra-coelomic injection with a mass-adjusted dose of Ringer's solution. The snake was promptly put back into its aquarium for exactly 30 min. At 30 min, the snake was removed and raced down the track as described above. Blood samples were taken and processed as described above. Two days later, the snakes were injected with one MAMU. This dose was selected relative to known resistance from previous investigations of this population (Brodie and Brodie, '90). After 30 minutes, snakes were raced and bled as described before.

Two days after TTX injections, all snakes were injected with mass-adjusted acetone solution because indoxacarb does not dissolve into Ringer's solution (control for TTX). There were no adverse effects of this injection. Four days after this control injection, snakes were injected with 0.167mg indoxacarb/gram snake (Indoxacarb (98.5% pure; ChemService, West Chester, PA)). Dosage was calculated using previous data showing that this concentration reduced snake speed by approximately 50% (LNL, unpublished). The concentration of INDOX in this study was not meant to be ecologically relevant, only elicit a similar behavioral response to TTX as a way to approximate comparable exposure. Snakes were raced and bled in the same manner as described for TTX.

Radioimmunoassay

Circulating corticosterone and testosterone levels were determined using a previously described protocol (French et al, '10; French et al, '06; Moore, '86). Samples were extracted using isooctane: ethyl acetate, dried, and resuspended in PBS buffer. Samples were assayed in duplicate and the mean of the two were used in analysis for both CORT (MP Biomedicals, Lot #3R3PB-19E) and TEST (Fitzgerald, Lot #01916). For each sample, we used an aliquot of the resuspended fractions to measure individual recoveries following extraction and chromatography. These recoveries were used to adjust final sample concentration values to account for any losses during these procedures. Standards of known value and negative controls were included in every assay as a reference to ensure accuracy. All samples were run in a single assay for each hormone. For CORT, intra-assay variation was 12.1% and TEST intra-assay variation was 15.3%.

Bactericidal Ability

We performed the bactericidal assay (BKA) to measure innate immune function, following the protocol outlined in French and Neuman-Lee ('12). Briefly, we combined a 1:4 dilution of plasma with CO_2-Independent media (Gibco, Grand Island, NY) plus 4nM L-glutamine (Sigma-Aldrich), and 10^5 CPU (colony producing unit) *Escherichia coli* (EPowerTM Microorganisms #0483E7, ATCC 8739, MicroBioLogics, St. Cloud, MN), and agar broth on a 96-well microplate. We calculated the background absorbance using BioRad xMark microplate reader. After a 12-hour incubation, we again read the

absorbance and calculated the bactericidal ability by dividing the mean absorbance for each sample (run in duplicate) by mean absorbance for the positive controls (containing only media and bacterial solution), and multiplying by 100. This provides the percent bacteria killed relative the positive controls. Negative controls (containing media only) were also run to ensure contamination was absent. Inter-assay variation between plates was 1.1%.

Statistics

For experiment one, we examined CORT, TEST, and BKA as explanatory variables and dose as the independent variable. We $\log_{10}$-transformed CORT to meet assumption of normality and homoscadicity and conducted an analysis of variance (ANOVA). For both TEST and BKA, we were unable to transform the data to meet assumptions of normality and therefore these tests were completed using the Wilcoxon Test. We also examined the CORT response between the first TTX injection for experiment 1 (1 MAMU, snakes did not race) and the first TTX injection for experiment 2 (1 MAMU, snakes did race). CORT had to be $\log_{10}$-transformed, and we analyzed the data using a t-test.

In experiment two, because the vehicle was different for the two chemicals (Ringer's solution for TTX and acetone for indoxacarb), we analyzed the response from baseline (vehicle injection) for each chemical instead of to each other. We conducted a t-test on $\log_{10}$-transformed CORT for TTX data to meet the assumptions of normality. We could not transform TEST or BKA data, therefore non-parametric Wilcoxon tests to

compare the baseline and post-TTX. For the indoxacarb data, both CORT and BKA were log$_{10}$-transformed and t-tests were conducted comparing baseline and post-indoxacarb data. The indoxacarb TEST could not be transformed and therefore a Wilcoxon test was performed.

RESULTS

Experiment 1: Dose-response of TTX

There was no relation between injection dose and any of the variables (Figure 6-1; log$_{10}$ CORT = $F_{(3,77)}$ = 1.12, p = 0.35; TEST = X^2 = 5.49, p = 0.14, d.f. = 3; BKA = X^2 = 0.37, p = 0.83, d.f. = 2). We were only able to measure BKA for baseline, 1 MAMU, and 3 MAMU (not 5 MAMU) due to limits on plasma volume.

When comparing the CORT response for the 1 MAMU doses for the snakes that ran vs. the snakes that did not run, we found that there was no difference (t = 1.39, p = 0.17).

Experiment 2: TTX and INDOX

There was no difference between baseline and post-TTX BKA (Figure 6-2; X^2 = 0.079, p = 0.79, d.f. = 1), CORT (Figure 3; t = 0.61, p = 0.55, d.f. = 30.77), or TEST (Figure 4; X^2 = 0.039, p = 0.84, d.f. = 1).

There was a difference between baseline and post-indoxacarb BKA (Figure 6-2; t = 3.32, p = 0.0024, d.f. 29.93) and CORT (Figure 6-3; t = 2.62, p = 0.013, d.f. 36.84). There was, however, no difference in TEST (Figure 6-4; X^2 = 0.23, p = 0.63, d.f. = 1).
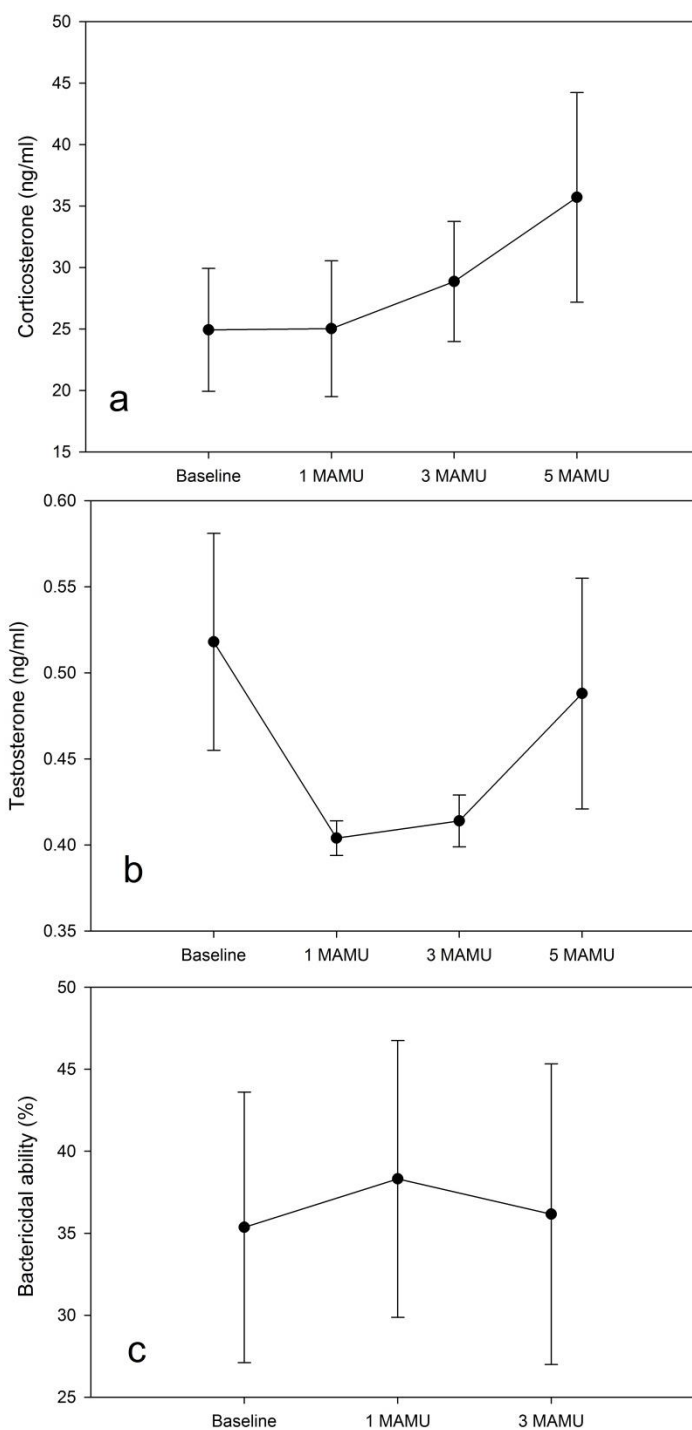
Figure 6-1. Physiological responses to increasing levels of TTX in Common gartersnakes (*Thamnophis sirtalis*). There was no differences in CORT (panel a), TEST (panel b), or BKA (panel c). Data are shown as raw values with $1 \pm$ standard error.

Figure 6-2. Bactericidal ability in Common Gartersnakes (*Thamnophis sirtalis*) after exposure to TTX or Indoxacarb. The pre-dose indicates the vehicle injection only (Ringer's solution for TTX and acetone for Indoxacarb). There is no difference between the vehicle injection and TTX, but bactericidal ability increased significantly between vehicle injection and Indoxacarb. Error bars indicate ± 1 standard error and the asterisk indicates significance ($\alpha = 0.05$).

Figure 6-3. Corticosterone in Common Gartersnakes (*Thamnophis sirtalis*) after exposure to TTX or Indoxacarb. The pre-dose indicates the vehicle injection only (Ringer's solution for TTX and acetone for Indoxacarb). There is no difference between the vehicle injection and TTX, but corticosterone increased significantly between vehicle injection and Indoxacarb. Error bars indicate ± 1 standard error and the asterisk indicates significance ($\alpha = 0.05$).

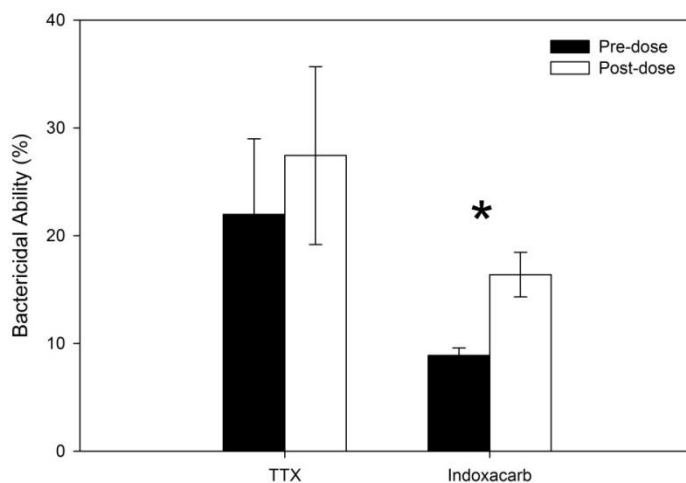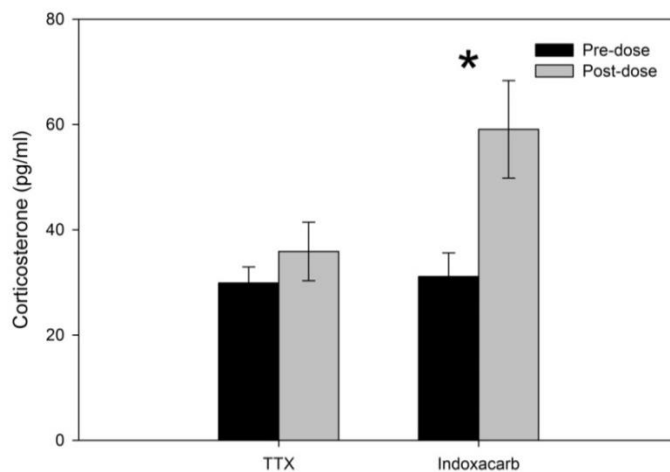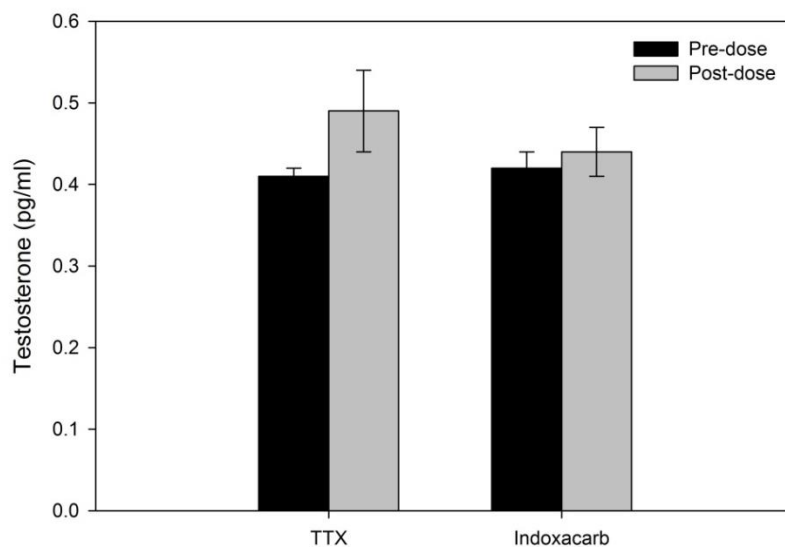Figure 6-4. Testosterone concentrations in Common Gartersnakes (*Thamnophis sirtalis*) after exposure to TTX or Indoxacarb. The pre-dose indicates the vehicle injection only (Ringer's solution for TTX and acetone for Indoxacarb). There were no differences between the vehicle and chemical injections. Error bars indicate ± 1 standard error.

DISCUSSION

This research indicates that being exposed to anthropogenic chemicals may elicit a greater response than exposure to chemicals with which an organism has an evolutionary history, even when the chemicals have a similar mechanism of action. In our first experiment, we showed that increasing doses of TTX did not elicit a significant physiological response among the parameters we measured. When examining the anthropogenic chemical INDOX in comparison with TTX, we were unable to directly compare the TTX and INDOX exposures. However, the increase from the baseline

(vehicle) in INDOX treatment for both CORT and BKA was significant but not in TTX treatment. Given the highly toxic nature of TTX, it is surprising that there was no CORT, TEST, or innate immune response to receiving any of the injections. This work highlights the need for determining how different chemicals affect the same model organism.

An increase in CORT in response to a chemical stressor is not unusual and has been documented in a variety of species and for many chemicals (Adams et al, '09; Franceschini et al, '08; Hopkins et al, '97; Sanders et al, '74). However, organisms do not always respond with an elevated CORT response. This lack of response may be associated with a depression of the HPA axis due to chronic stress (Dhabhar, '09; Rich and Romero, '05), lack of perception of a threat (Cockrem, '07; Neuman-Lee et al, '15), or blocking of the HPA axis by the chemical's action (Gendron et al, '97; Ilan and Yaron. '80). Further, it seems that these responses may be dependent upon context-dependent factors such as age, sex, and reproductive status (Bechshoft et al, '15; Crespi et al, '13; Lattin et al, '12). While we cannot rule out that TTX itself blocks action by the HPA axis, the mechanism of action does not appear to interfere with this process (Narahashi, '01). Further, a recent study showed that females (but not males) elevated their CORT in response to exposure to TTX, which indicates that TTX itself likely does not block the process (Neuman-Lee et al, In Press).

It was surprising, however, that these snakes had no response to TTX, but mounted a strong response to INDOX given the similar mechanism of action. However, this could be explained by two non-mutually exclusive reasons. First, the molecular structure of INDOX is very different from that of TTX (McCann et al, '01; Narahashi,

'01), and thus the snake's body likely processes the toxin in a different manner. Unfortunately, there is limited reptilian toxicokinetic data for TTX (Williams et al, '12) and none for INDOX to either substantiate or refute this possibility. Second, the evolutionary history that snakes share with TTX exposure (through eating *Taricha granulosa*) may reduce responses to TTX exposure. While these snakes tested were naïve to TTX and do not co-exist with *T. granulosa*, they still have some resistance to TTX (Feldman et al, '09). Other evolutionary adaptations, such as adaptations to the HPA axis, immune function, or reproduction have not yet been examined and therefore cannot be ruled out as a possible reason for the lack of response to TTX.

The innate immune response also followed the same pattern as CORT. We saw a marked increase in bactericidal ability after INDOX exposure, while there was no change after TTX exposure. This is likely due to the corresponding elevation in CORT. During many acute stressors, the secretion of CORT is correlated with an increase in immune activity (Dhabhar, '09; Dhabhar and McEwen, '97). While not a universal pattern, this relationship is thought to arise as a protective mechanism (Dhabhar et al, '12; Sapolsky et al, '00) and has been seen in previous studies of acute stress exposure (Dhabhar and McEwen, '97; Martin, '09).

Neither TTX nor INDOX appeared to affect TEST secretion. There is a possibility that because TEST levels were already low, there is no appreciable action that can either block or increase TEST secretion. However, while research on other populations of *T. sirtalis* has indicated that TEST remains low during the spring and through mating (Moore et al, '67), long-term data on this population in Utah indicates

that many snakes do have higher circulating TEST during the spring (LNL, unpublished data). We therefore hypothesize that neither INDOX nor TTX at these levels has an impact on TEST. Snakes from another population also responded similarly to varying levels of TTX by not elevating or decreasing their circulating TEST concentrations (Neuman-Lee et al, In Press).

Because there are no ecological data on the levels of INDOX in the environment, it is not possible to determine if the amount that was given is ecologically relevant. Therefore, these results should not be interpreted as a challenge that wild snakes would necessarily encounter. However, with the increased amount of chemicals entering the environment, as well as other anthropogenic pressures, understanding how reptiles respond to chemicals will allow the regulatory community to better manage populations. As a taxonomic group, reptiles remain imperiled (Gibbons et al., 2000) and often overlooked in ecotoxicological studies. Snakes in particular face a new, potentially devastating threat of the infectious disease Snake Fungal Disease (SFD; (Allender et al, '11; Sutherland et al, '14)), and any challenge which alters their immunity and physiology puts them at increased risk. Therefore, determining as much about their physiology and response to anthropogenic factors is critical.

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CHAPTER 7

PHYSIOLOGICAL EFFECTS OF POLYBROMINATED DIPHENYL ETHER (PBDE-

47) ON PREGNANT GARTERSNAKES AND RESULTING OFFSPRING[6]

Polybrominated diphenyl ethers (PBDEs) are used as a flame retardants and are a

persistent contaminant found in virtually every environment and organism sampled to

date, including humans. There is growing evidence that PBDEs are the source of thyroid,

neurodevelopmental, and reproductive toxicity. Yet little work has focused on how this

pervasive contaminant may influence the reproduction and physiology of non-traditional

model species. This is especially critical because in many cases non-model species, such

as reptiles, are most likely to come into contact with PBDEs in nature. We tested how

short-term, repeated exposure to the PBDE congener BDE-47 during pregnancy affected

physiological processes in pregnant female gartersnakes (thyroid follicular height,

bactericidal ability, stress responsiveness, reproductive output, and tendency to terminate

pregnancy) and their resulting offspring (levels of corticosterone, bactericidal ability, and

size differences). We found potential effects of BDE-47 on both the mother, such as

increased size and higher thyroid follicular height, and her offspring (increased size),

suggesting the effects on physiological function of PBDEs do indeed extend beyond the

traditional rodent models.

INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) are compounds used as flame retardants for industrial, commercial, and residential purposes (Birnbaum and Staskal, 2004). Concentrations of PDBEs have increased in human tissue by a factor of 100 since their first wide-spread use in the 1970s (Hites, 2004). Although companies began to phase out use of PBDEs in 2004, they are still released into the environment at a high rate through dissociation, disposal, and incineration (Gullett et al., 2009; Hale et al., 2003; Xu et al., 2009).

PBDEs have been found in high concentrations in nearly every environment surveyed, including atmosphere (Strandberg et al., 2001; Su et al., 2007), water (Hale et al., 2003; Qiu et al., 2010), and substrates such as soil and sediment, where they remain stable and long-lasting (Hale et al., 2003; Hites, 2004). Great attention has focused on PBDEs because of their prevalence in human blood and breast milk (Bi et al., 2006; Hooper and McDonald, 2000; Lorber, 2008; She et al., 2007). Numerous studies have documented presence and bioaccumulation of PBDEs in a variety of animals, including seals, sea birds, trout, frogs, invertebrates, raptors, and turtle eggs (Hale et al., 2003; Hall et al., 2003; reviewed in Law et al., 2003; Venier et al., 2010; Weijs et al., 2010) and potential for long-range atmospheric transport, especially in the lower brominated class of PBDEs, such as 2,2',4,4'-tetrabromodiphenyl ether (BDE-47; Boon et al., 2002; Gouin and Harner, 2003).

Although presence of PBDEs in wildlife is evident, relatively little research has

been conducted on functional effects of PBDE exposure. There is evidence that the

thyroid gland and its hormonal products are impacted by exposure to PBDEs (Costa et

al., 2014; Hallgren and Darnerud, 2002; McDonald, 2002) which is likely due to the

structural similarity of PBDEs to thyroxine (T_4; Meerts et al., 2000). Lower brominated

PBDEs compete with and often displace T_4 when binding to the critical thyroid transport

protein transthyretin (TTR; Meerts et al., 2000). This competitive binding may result in

hypothyroidism because when target cells do not receive thyroid hormones, hypertrophy

of the thyroid follicular cells can occur (Capen and Martin, 1989). Contaminant-induced

hypothyroidism can affect a suite of characters, including metabolism, brain

development, somatic growth, and development of reproductive organs (Cooke et al.,

2004; Parrott et al., 1961; Rivera and Lock, 2008) in mammals (de Wit, 2002; Hallgren et

al., 2001; Zhou et al., 2001), birds (Fernie et al., 2005b), fish (reviewed in Brown et al.,

2004 and Carr and Patiño, 2011), amphibians (reviewed in Carr and Patiño, 2011) and

likely other vertebrates, as thyroid hormones are highly conserved across vertebrate taxa

(Hulbert and Else, 1981).

Due to the known effects on the thyroid, PBDEs may potentially affect other

physiological processes. One key response in vertebrates to environmental challenges

including contaminants is energy metabolism and secretion of glucocorticoids (GCs)

through activation of the hypothalamic-pituitary-adrenal-axis (HPA; Wingfield and

Romero, 2001; Franceschini et al., 2008; Gunderson et al., 2003; Hopkins et al., 1997;

Lorenzen et al., 1999; Pruett et al., 2003; Romero and Wikelski, 2002; Sanders et al.,

1974). Glucocorticoids, such as corticosterone (CORT), are important mediators in mobilization of energy during stress (Wingfield and Romero, 2001). Given that wild animals have limited energy stores, investing energy into coping with a stressor requires trade-offs with other physiological processes, such as reproduction and immune function (Bremner and Vermetten, 2001; Cooke et al., 2004; French et al., 2007; Suter and Schwartz, 1985; Tilbrook et al., 1999; Tilbrook et al., 2000). Therefore, GCs may serve as an important link between contamination and the health of the individual (Wingfield and Sapolsky, 2003).

Reproduction is one of the most energetically-costly life history stages for an individual (Gittleman and Thompson, 1988). Therefore, required increases in energetic requirements needed to cope with other stressful events, such as contaminant exposure, may cause a decrease or cessation in reproduction (Wingfield and Sapolsky, 2003) through spontaneous abortion or resorption of follicles and embryos (Clark et al., 1993; Mendola et al., 2008). Studies on rodent models have also indicated direct deleterious effects of BDE-47 on reproduction (Lilienthal et al., 2006; Talsness et al., 2008) and developing embryos (reviewed in Costa et al., 2008); however, wildlife have not been well investigated, despite a growing body of evidence suggesting negative effects in fish (Arkoosh et al., 2010; Lema et al., 2008; Muirhead et al., 2005), mammals (Hall et al., 2003), birds (Fernie et al., 2008), and amphibians (Van Schmidt et al., 2012).

When an organism is undergoing chronic stress and has an even more limited pool of resources due to physiological processes such as reproduction, measuring immune function is a critical component in determining the consequences of this stress.

PBDEs have been implicated in decreased immune function in both birds (Fernie et al., 2005a) and humans (Leijs et al., 2009), although some studies show no immune consequences of PBDE exposure (Fernlöf et al., 1997). Innate immune function is a highly effective measure of immunocompetence because it relies on a rapid response to virtually all foreign pathogens (Janeway et al., 2001). Further, vertebrates have similar innate immune function, while humoral and cell-mediated responses differ among taxa (Zimmerman et al., 2010).

Populations of reptiles are declining rapidly around the world, yet we know little about how reptiles respond to contamination (Gibbons et al., 2000; Hopkins, 2000; Sparling et al., 2010). Ecotoxicological research involving squamates (snakes and lizards) is limited (Hopkins et al., 1999; Murray et al., 2010; Ohlendorf et al., 1988). Snakes are important as ecotoxicological models because they are found in a wide variety of ecosystems, they are large enough for use in physiological tests, exhibit high site fidelity, are long-lived, and many species exist in abundance in contaminated environments (Beaupre and Douglas, 2009; Hopkins et al., 2002; Hopkins et al., 1999; Hopkins, 2000; Neuman-Lee et al., 2014). We studied the Western Terrestrial Gartersnake (*Thamnophis elegans*), which is found throughout the western United States and Canada (Rossman et al., 1996) across a diversity of different landscapes, are viviparous (live-bearing), dietary generalists, and their basic physiology has been well-studied, providing an important knowledge base (Moore et al., 2000; Robert and Bronikowski, 2010; Rossman et al., 1996; Sparkman and Palacios, 2009).

To determine potential effects of chronic BDE-47 ingestion on snakes, we examined a suite of physiological endpoints that influence individual health and reproductive capabilities in adult females. We quantified effects of BDE-47 on mass, thyroid morphology, stress-reactivity, ability to maintain gravidity throughout the reproductive season, and innate immunocompetence in adult female gartersnakes and size at birth, sex ratio, CORT levels, and innate immunocompetence in their developing neonates. We hypothesized that female snakes exposed to BDE-47 would show thyroid hypertrophy and chronically elevated CORT, which would correlate with lowered stress-reactivity, reproductive investment, and immune function. We also hypothesized that neonates exposed to BDE-47 in utero would be smaller, have higher CORT levels, and decreased immunocompetence.

METHODS

Animal Collection and Care

Female gartersnakes (*Thamnophis elegans*) were collected in Cache Valley, Utah in April and May of 2012. We transported snakes to Utah State University in opaque cloth bags and housed them individually in 37.8-liter glass aquaria. Each aquarium was lined with newspaper and had a plastic hide with moist sphagnum moss. We supplied deionized water in glass dishes. The room was kept at 26°C on a 12:12 on:off light cycle. Snakes were weighed every two weeks and given 20% of their body weight in thawed mice, alternating with weeks in which they were dosed. The mass of food consumed was

recorded and uneaten mice were removed after 24 hours. We confirmed large, developing follicles in snakes in late May (directly prior to dosing) using an ultrasound (Sonosite, MicroMaxx) and palpitation. We determined termination of pregnancy (resorption of follicles) by the complete absence of birthing (stillborn, unfertilized, or live born).

Snakes were randomly divided into one of two treatment groups (BDE-47 and vehicle control). To simulate ingestion of contaminated food items, snakes were weighed then orally gavaged with either corn oil (n = 10; control) or 2,2',4,4'-tetrabromodiphenyl ether (BDE-47, Sigma Aldrich, St. Louis, MO) in corn oil (n = 9) every two weeks. Snakes receiving BDE-47 were given 50 ng/g body weight each dosing period such that each female received 300 ng/g body weight by the end of the study. The chosen dose fell midway within the range of doses found in field settings in wild organisms (Hale et al., 2003; Law et al., 2003). Because no studies have yet been published on concentrations of PBDEs in wild snakes, we selected this dose based on studies conducted by Fernie et al. (2005a, 2005b) in which physiological effects on kestrels were observed. We, however, modified the dosing schedule slightly to better accommodate snakes' less frequent feeding strategy.

Bi-weekly Blood Sampling and Stress-Reactivity Test

We took a blood sample every other week throughout the reproductive season (beginning in June) until August, such that there were six samples for each female. For the stress-reactivity test at the end of the experiment, post-parturition (or after the

termination of pregnancy was confirmed) in August, we obtained the first sample as described below within three minutes (baseline). We then placed the snake individually in an opaque bag, and took a second blood sample after 30 minutes (stress-induced).

For all blood sampling, we obtained approximately 200 ul of blood via the caudal vein using a sterile syringe within three minutes of removing the snake from its cage. Samples were placed on ice for less than 1 hr and centrifuged at 1000 g for 10 min. Plasma was separated from the red blood cells and stored at -80°C until further processing (radioimmunoassay and bacterial killing assay; see below).

After the females had given birth or terminated pregnancy was confirmed, all snakes were sacrificed using rapid decapitation. The thyroid was removed immediately and preserved for histological analysis (see below). All procedures were approved by the Utah State University IACUC (Protocol #2299).

Neonates

We measured neonates within 48 hrs. after birth (mass, snout-vent-length (SVL)). Sex was determined by cloacal probe. The neonates were housed for one month with one sibling in a newspaper-lined opaque plastic container with a thermal gradient, 12:12 hour on:off light cycle, and water dish. After one month, each individual was sacrificed using rapid decapitation and the trunk blood was collected and pooled within sex and litter for further analyses (see below). Pooling blood was necessary to acquire the necessary volumes for running the assays. Blood was processed as described above.

Thyroid Histology

Histological procedures followed the description of Carr et al. (2003). Adult thyroid glands were removed immediately after euthanizing and placed in 10% buffered formalin and stored until preparation (BDE-47: n = 6, control: n = 4). Individual thyroid glands were used to prepare blocks of paraffin for thyroid histopathology. Prior to sectioning, blocks were treated with a softening agent (Mollifex, Harleco). Sections were cut at 10μm thickness, processed for routine paraffin embedding, and stained with hematoxylin and eosin. Histological sections through the middle of thyroid glands were chosen for analysis, and at least nine photos from three different follicles per gland were analyzed by a naïve reader. Digital images of the thyroid follicles were taken with a Nikon digital camera (DS-Fi1; Tokyo, Japan) attached to a compound microscope (Nikon Eclipse 557). Measurements were conducted digitally using ImageJ (National Institutes of Health). Three follicular epithelial cells were measured in each of at least three follicles per snake thyroid. Mean cell height was calculated for each follicle, and the mean of all measured follicles was determined for each sample.

Radioimmunoassay (RIA)

Circulating hormone levels were determined using a previously described protocol (French et al., 2010; French et al., 2006; Moore, 1986). Plasma was separated from the cells via centrifugation and stored at -20 °C until assayed. Samples were extracted using isooctane: ethyl acetate, dried, and resuspended in PBS buffer. Samples were assayed in duplicate for CORT (MP Biomedicals, Lot #3R3PB-19E). For each

sample, we used an aliquot of the resuspended fractions to measure individual recoveries following extraction and chromatography. These recoveries were used to adjust final sample concentration values to account for any losses during these procedures. Standards of known value and negative controls were included in every assay as a reference to ensure accuracy. All samples were run in a single assay. Intra-assay variation was 9.8% and accuracy was 88.8%.

Innate Immunocompetence

We performed the bactericidal assay (BKA) to measure innate immune function, following the protocol outlined in French and Neuman-Lee (2012). Briefly, we combined a 1:4 dilution with CO_2-Independent media (Gibco, Grand Island, NY) plus 4nM L-glutamine (Sigma-Aldrich), and 10^5 CPU (colony producing unit) *Escherichia coli* (EPowerTM Microorganisms #0483E7, ATCC 8739, MicroBioLogics, St. Cloud, MN), and agar broth on a 96-well microplate. We calculated the background absorbance using BioRad xMark microplate reader. After a 12-hour incubation, we again read the absorbance and calculated the bactericidal ability by dividing the mean absorbance for each sample (run in duplicate) by mean absorbance for the positive controls (containing only media and bacterial solution), and multiplying by 100. This provides the percent bacteria killed relative the positive controls. Negative controls (containing media only) were also run to ensure contamination was absent. Inter-assay variation between plates was 8.9%.

Statistical Analyses

We assessed all variables for normality. The following variables had to be log-transformed to meet the assumption of normality: all CORT concentrations (biweekly samples and stress-reactivity test), mass of adult female snakes over time, and average neonate mass. The bactericidal ability over time was rank transformed. The bactericidal ability for adult females for the stress-reactivity test (baseline and stress-induced) was unable to be transformed to meet normality so non-parametric analyses were conducted.

The mean of all measured thyroid follicular cell heights for each snake was calculated, and the two treatment groups were compared using a t-test. One of the control females had a highly variable follicle size compared to all other individuals and her average was more than two standard deviations away from the mean. Therefore, we ran t-tests both with and without her follicle size.

A repeated measures analysis of variance (rmANOVA) was performed for mass, log CORT, and bactericidal ability. We regressed bactericidal ability and log CORT to determine the relationship between the two measures over the course of the summer. To assess the stress-reactivity at the end of the experiment, a rmANOVA was conducted on CORT and bactericidal ability using baseline and stress-induced samples as time points. Additionally, we conducted a t-test on the log CORT baseline and log CORT stress-induced samples to compare each point between treatments. For bactericidal ability, a Wilcoxon Rank-Sums test was used. The ΔCORT and Δ bactericidal ability were assessed using a t-test to compare between BDE-47 and control animals.

We measured the incidence of terminating reproduction using a contingency test. Given that some snakes in both treatment and control groups terminated reproduction, and that reproductive output is often related to our other physiological measures, we separated the females that completed their pregnancy and those that did not. All of the above analyses were conducted for both of these groups.

For animals that did not terminate pregnancy, we calculated the mean of each litter for each of the following parameters: total neonate mass, average neonate mass, number of neonates born alive, sex, CORT, and bactericidal ability. To test if there were differences between neonates exposed to BDE-47 or exposed to the control, total neonate mass and log average neonate mass were compared using an ANCOVA with the mother's weight as a covariate. T-tests were used to determine differences between BDE-47 and control groups in terms of number of neonates born live, percent female, CORT, and bactericidal ability. Statistical significance was set at $p \leq 0.05$.

RESULTS

Adults

Average thyroid follicular height was 23% higher in females exposed to BDE-47 compared to control females (Figure 1, $t = 3.51$, $p = 0.0135$ (excluded control individual); $t = 1.43$, $p = 0.20$ (included control individual). 56% (5 out of 9) of PBDE-treated females terminated their pregnancy, while 30% (3 out of 10) control females did the same (Figure 2, $X^2 = 1.28$, $p = 0.26$).
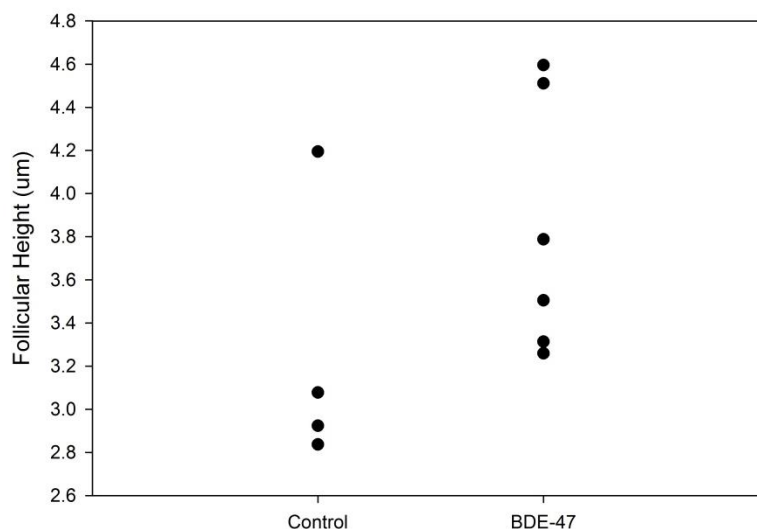
Fig. 7-1. Average thyroid follicular cell height (um) for female gartersnakes (*Thamnophis elegans*) exposed to BDE-47 (n = 6) or vehicle only (n = 4). One control female had a significantly higher mean cell height (> 2 SD from the mean). The cell heights were also highly variable and therefore statistical tests were conducted with and without her (t = 3.51, p = 0.0135 (excluded control individual); t = 1.43, p = 0.20 (included control individual)).
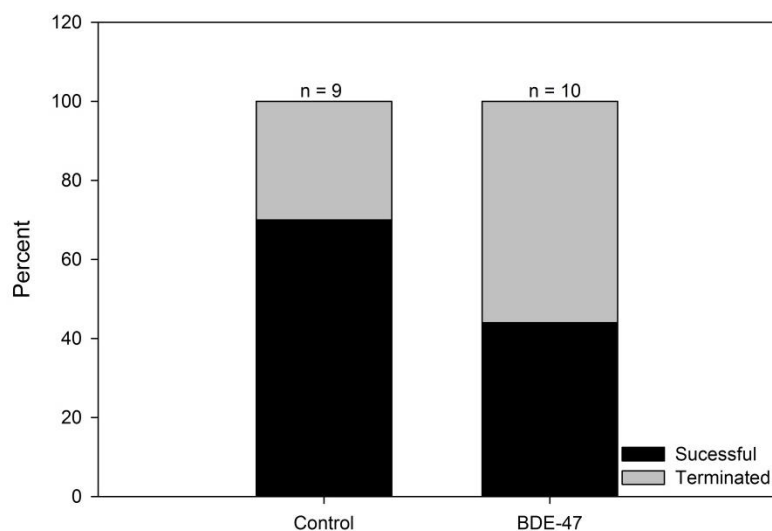

Fig. 7-2. The percent of successful vs. terminated pregnancies in BDE-47 or vehicle treated female gartersnakes (*Thamnophis elegans*; $X^2 = 1.28$, p = 0.26).

There was a pronounced difference in mass over the gestation period between the two treatment groups in females that gave birth (Figure 3; $F_{1,1} = 11.71$, p = 0.001) with BDE-47 females being heavier, despite controlling for food intake. All BDE-47 exposed snakes (both pregnant and resorbing females) weighed slightly more over time (Figure 3, $F_{1,1} = 2.64$, p = 0.11).

There were no differences between the bi-monthly levels of CORT between the two treatment groups over the gestation period, regardless of whether or not the females gave birth (p > 0.3). Additionally, BDE-47 treatment did not alter the CORT levels in the stress-reactivity challenge, although the baseline levels of CORT were slightly higher in control animals (Figure 4, t = -2.01, p = 0.069).
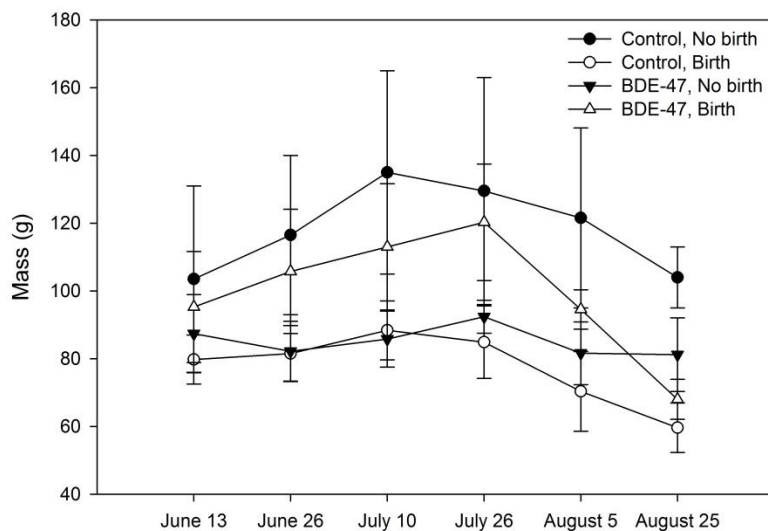

Fig. 7-3. Changes in mass of gartersnake females (*Thamnophis elegans*) taken bi-weekly over gestational period when treated with either BDE-47 or vehicle. Some individuals terminated pregnancy and therefore comparisons are made overall ($F_{1,1} = 2.64$, p = 0.11) and between the females that reproduced only (Figure 3; $F_{1,1} = 11.71$, p = 0.001).

Bactericidal ability throughout the gestation period also did not differ between BDE-47-treated females and control females, regardless of reproductive success (p > 0.2). The stress-reactivity challenge did, however, demonstrate that among females that did not give birth the control females had a lower bactericidal ability than the BDE-47 females (Figure 5, $X^{2=}$ 5.68, p = 0.017).
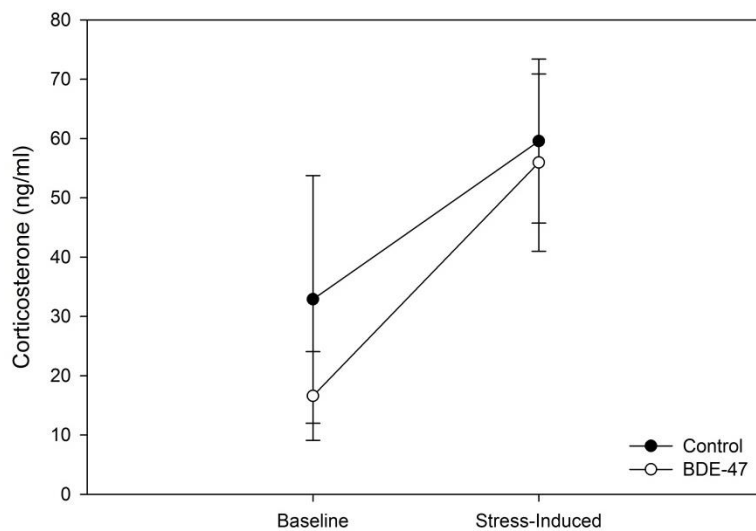

Fig. 7-4. Stress-reactivity as measured by corticosterone levels between BDE-47 (n = 6) and vehicle (n= 7) treated gartersnake females (*Thamnophis elegans*). Control females have higher baseline corticosterone levels (p = 0.069), but there is no difference in stress-induced levels or the change between the two (p > 0.7). Bars indicate ±1 standard error.
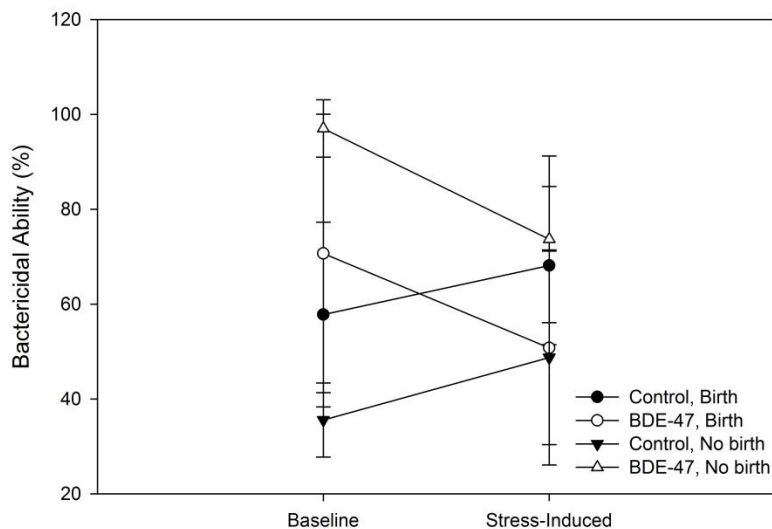
Fig. 7-5. Stress reactivity as measured by bactericidal ability in female gartersnakes (*Thamnophis elegans*). BDE-47 females had a higher baseline bactericidal ability (p = 0.096) than control females (Control n = 9, BDE-47 n = 8). The lowest bactericidal ability was demonstrated in control females that terminated their pregnancy and was lower than the BDE-47 females that terminated pregnancy (p = 0.017, Control n = 3, BDE-47 n = 5). There was no difference between the two treatment groups in females that gave birth (p > 0.7).

Neonates

Neonates born to BDE-47 treated females were, on average, larger in terms of both mass and SVL than those born to control females (Table 1; mass: $F_{1,9}$ = 9.26, p = 0.014). However, the total neonate mass per clutch was not different between treatment groups ($F_{1,9}$ = 0.23, p = 0.64). Number of neonates born live, the sex ratio, CORT concentrations, and bactericidal ability were also not affected by BDE-47 exposure in utero (Table 1, p > 0.3).

Table 7-1

Morphometric and physiological measurements of neonates born to chronically exposed BDE-47 female gartersnakes (*Thamnophis elegans*) (Control n = 7 females; BDE-47 n = 4 females).

	Treatment	Averages ± Standard Error	Statistics
Number Neonates Born Live	Control	9.57 ± 1.62	$F_{1,9} = 0.13, P = 0.73$
	BDE-47	10.50 ± 1.89	
Sex Ratio (F:M)	Control	0.52 ± 0.06	$F_{1,9} = 1.23, P = 0.30$
	BDE-47	0.39 ± 0.10	
Combined mass of all neonates (g)	Control	18.61 ± 3.50	$F_{1,9} = 0.23, P = 0.64$
	BDE-47	27.55 ± 6.07	
Average Neonate Mass (g)	Control	1.94 ± 0.13	$F_{1,9} = 9.26, P = 0.014*$
	BDE-47	2.58 ± 0.17	
Average Neonate SVL (mm)	Control	171.54 ± 3.93	$F1,9 = 8.47, P = 0.02*$
	BDE-47	187.37 ± 2.96	
Corticosterone (ng/ml)	Control	46.49 ± 20.97	$F_{1,9} = 0.14, P = 0.72$
	BDE-47	50.98 ± 40.87	
Bactericidal Ability (%)	Control	27.13 ± 3.37	$F_{1,9} = 0.30, P = 0.59$
	BDE-47	23.90 ± 5.07	

DISCUSSION

We found several important trends in snakes exposed to BDE-47 that suggest this contaminant may affect snakes. While these findings were not all statistically significant ($\alpha \leq 0.05$), they are potentially biologically significant. Thyroid follicular cell height was greater in BDE-47 individuals (although one control animal had comparable cell sizes). Additionally, over half of the BDE-47-treated snakes terminated their pregnancy during their gestational period and therefore did not reproduce. The BDE-47 females that did give birth were heavier than control females and, gave birth to larger neonates compared to control females (corrected for body size). Further, the BDE-47 females had lower

baseline CORT during the stress-reactivity test. And, taken together, these trends of

hypertrophy of thyroid follicular cells, reduced reproduction, increased mass, and

lowered baseline CORT may indicate that snakes are at some risk during and after

chronic exposure to BDE-47. We did not however, observe a significant impact of BDE-

47 on hormone levels or immunity in pregnant adult females or neonates.

Adults

Our study provides support that low levels of BDE-47 may disrupt the thyroid

axis by increasing or decreasing T4 levels, causing thyroid hypertrophy in wild animals,

potentially resulting in our observed negative impacts. The hypertrophy of the thyroid

follicular cells may indicate elevated levels of circulating T_4, which is often correlated

with altered physiology, such as impaired reproduction, neonatal development, and sex

hormone production (Cooke et al., 2004; Rivera and Lock, 2008). Alternatively, follicle

cell hypertrophy may reflect an inhibition of T_4 synthesis or other disruption to negative

feedback regulation of the upstream hormones thyrotropin releasing hormone (TRH) and

thyroid stimulating hormone (TSH). Abundant evidence indicates that contaminants

which inhibit T_4 and T_3 synthesis cause follicle cell hypertrophy. For example, exposure

of developing amphibians to perchlorate (Goleman et al., 2002), an I^- uptake inhibitor, or

methimazole (Tietge et al., 2010), a thyroperoxidase blocker, both lead to follicle cell

hypertrophy in exposed animals due to inhibition of thyroid hormone synthesis and

compensatory increases in TSH release. While we do not know the mechanism of

hypertrophy development in snakes, other studies have demonstrated possible reasons for

this finding. Due to their structural similarities, PBDEs mimic T_4 and subsequently bind competitively to the transport protein transthyretin (TTR; McDonald, 2002; Meerts et al., 2000), making it impossible for T_4 to bind to target cells (Hallgren and Darnerud, 2002). When the target cells do not receive the active T_4, the thyroid follicular cells continue to produce and excrete T_4, which, if prolonged, can cause hypertrophy due to a lack of negative feedback on the thyroid gland (Hallgren and Darnerud, 2002), although different tissues may be able to mediate these fluctuations by altering deiodinase expression (reviewed in Köhrle, 1999 and Yen, 2001). Contaminant-induced disruption of the thyroid can impair downstream effects on growth, reproduction, development, and survival (reviewed in Boas et al., 2006; Colborn, 2002; Tabb and Blumberg, 2006).

Reduced reproduction in any organism can have dramatic effects on population persistence and growth. Therefore, if more females are unable to maintain their pregnancy while chronically exposed to low levels of BDE-47, population levels may decrease. This is especially true in our study given that even control females do not always maintain their pregnancy. Terminating reproduction is documented in response to a chronic stressor and/or contaminant exposure (Wingfield and Sapolsky, 2003). However, the prevalence of aborting or resorbing follicles in viviparous snakes is still debated. These snakes apparently produce more follicles than the number of offspring they produce. Yet it remains unknown as to the reason behind this seemingly "extra" investment or the ultimate fate of the extra follicular tissue (Blackburn et al., 2003; Bonnet et al., 2008). In this study, it is likely that large follicles were resorbed by the females, as we found no evidence of follicle tissue in the cages or oviducts of necropsied

snakes and ultrasounding revealed that the follicles had disappeared. We also do not know if the follicles had been fertilized. Captivity itself could have influenced reproductive success as it can be considered a chronic stressor (Morgan and Tromborg, 2007) and, while we do not know the reproductive capabilities of snakes from this population, all of the control and BDE-47 females were kept in identical conditions and were thus exposed to the same stressors in captivity with the exception of the BDE-47 treatment. Interestingly, the control females that completely terminated their reproduction also had a significantly lower bactericidal ability. This may indicate that these females were in an energetically or otherwise compromised state relative to others in the study.

The baseline CORT levels during the stress-reactivity test provided some evidence that BDE-47 may affect the HPA axis. Treated females had lower CORT concentrations than the control females, potentially indicating energetic limitations after the chronic BDE-47 exposure. Throughout the gestational period, there was no difference in CORT levels which may be due to the pronounced effects of reproduction on circulating CORT levels (Love et al., 2004), overwhelming any treatment effects, although the mean of the control females was consistently higher than that of the BDE-47 treated animals. There were also no differences between CORT levels in reproducing and non-reproducing females. However, all of these results should be viewed with caution because reproduction is known to impact CORT levels in organisms and the majority of the BDE-47 treated animals did not reproduce.

Innate immunocompetence was apparently impacted by chronic BDE-47 dosing, although it increased instead of the predicted decrease. Other studies have shown

negative effects of PBDE exposure on immune function in bird, fish, and mammalian

models (Arkoosh et al., 2010; Frouin et al., 2010; Martin et al., 2007; Thuvander and

Darnerud, 1999). For example, Fernie et al. (2005a) examined the effects of young chicks

ingesting PBDEs and found that phytohaemagglutinin (PHA) response increased while

the spleen mass, somatic index, and number of germinal centers decreased in exposed

birds. PBDEs have also been implicated in increasing oxidative damage in many tissues

(reviewed in Costa et al., 2008). However, Fernlöf et al. (1997) found no relationship

between PBDE exposure and immune function. Yet, the differences between our findings

and other studies may be partly influenced by the dissimilarity between reptile and

mammalian/avian immune systems (Zimmerman et al., 2010).

Neonates

Offspring growth and development is critical to ultimate female reproductive

success. Thus we also examined the effects on neonate size and sex after being exposed

in utero. Previous work in frogs and birds has shown that females do transfer BDE-47 to

their eggs (Liu et al., 2011; Verreault et al., 2006), and thus the offspring in this study

were likely exposed. Our major finding was that the average mass of neonates tended to

be larger in the BDE-47 treated animals, which is similar to findings in zebrafish exposed

to the thyroid disruptor perchlorate during development (Mukhi and Patiño, 2007). Yet a

study also conducted on zebrafish exposure during development to BDE-47 found that

high concentrations yielded smaller individuals (Lema et al., 2008). However, larger

neonate size does not necessarily indicate a higher overall quality. In some instances,

larger neonates often have a greater chance of survival (Janzen et al. 2000; Kissner and Weatherhead 2005), yet this is not always the case and seems to vary in different contexts (Congdon et al. 1999; Filoramo and Janzen 2002; Litvak and Leggett 1992).

Our additional measures of neonates (number of neonates born live, the sex ratio, CORT concentrations, and bactericidal ability) demonstrated no other effects of BDE-47 treatment, which is similar to other studies in rodents (Zhou et al., 2002). Other work that has been conducted on exposure during neonatal development has demonstrated that contaminants may have profound effects on the individual that are manifested later in life. Brain development and function in particular may be greatly affected by BDE-47 exposure as a neonate (Costa et al., 2008). Studies have indicated that exposure to BDE-47 as a neonate is correlated with severe behavioral changes (Chou et al., 2010; Eriksson et al., 2001), including alterations in the function of the hippocampus (Dingemans et al., 2007), decreased physical and mental development (Herbstman et al., 2010), and decreased reproductive capability (Talsness et al., 2008). Many of these studies examine the exposed individuals at time points much later than the exposure. In contrast, our study only quantified morphological and physiological parameters soon after birth. Allowing the neonates to mature may have elucidated long-term effects that were not detectable immediately.

Finally, the concentrations of BDE-47, while environmentally realistic, were highly conservative. While we do not know the levels of BDE-47 in the wild snakes or the local prey, other studies on potential prey items for gartersnakes, such as fishes and small mammals, frequently have BDE-47 concentrations more than double 50 ng/g

(Darnerud et al., 2001; de Wit, 2002; Hale et al., 2003). These samples have been obtained from a wide range of habitats and species and, even if the population from which these individuals came is not contaminated, gartersnakes are found throughout North America (Rossman et al., 1996) and are likely consuming prey with some level of BDE-47. Additionally, a long-range atmospheric transfer model predicts that in the case of BDE-47 up to 50% of the released chemical may be stored in deciduous trees, making even herbivorous organisms likely to be exposed (Gouin and Harner, 2003). This increases the probability that wild snakes will consume items that have higher levels BDE-47 in their tissue.

In wild populations, organisms are exposed to a multitude of environmental contaminants and stressors that likely affect individuals differently when combined. Exposure to multiple congeners of PBDEs has been shown to have greater negative impacts than exposure to only one (Tagliaferri et al., 2010). Exposure to BDE-47 in conjunction with both hypoxia (Chan et al., 2014) and polychlorinated biphenyls (PCBs; He et al., 2009) is also more detrimental than exposure to only one of these environmental factors. More work is clearly needed to examine the effects of BDE-47 and other PBDEs in conjunction with the myriad of other environmental contaminants.

Conclusions

This study provides evidence that BDE-47 may impact many physiological processes, including endocrine function (thyroid and HPA axis) and reproduction. The possibility for these effects, such as decreased reproduction and thyroid dysfunction, of

BDE-47 contamination in non-model organisms is critical information for government organizations and policy makers to consider. Reptiles are declining at an alarming rate (Gibbons et al., 2000), and while there are likely many reasons for this decline, the pressures of toxic contamination on reptilian populations has remained very understudied and may be a contributor (Hopkins, 2000; Sparling et al., 2010). In fact, in most ecological risk assessments (ERAs), birds are evaluated and used as a proxy for reptilian species (Environmental Protection Agency, 2004; Moore et al., 2003; Sample et al., 1997). However, new models show that reptiles may be as susceptible as or even more susceptible to contamination than traditional models due to direct dermal exposure in both terrestrial and aquatic environments (Weir et al., 2010). Thus it is critical to determine the effects of contaminants on these non-model, wild organisms in order to further our understanding of ecosystem pressures.

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CHAPTER 8

FOOD RESTRICTION AND CHRONIC STRESS ALTER ENERGY USE AND AFFECT

IMMUNITY IN AN INFREQUENT FEEDER[7]

Glucocorticoids are important mediators of energy utilization for key physiological processes, including immune function. Much work has focused on the effects of energy limitation and stress for key physiological processes such as reproduction and immunity. However, it is unclear how stress alters energy use across different energy states, and the physiological ramifications of such effects are even less clear. In this study, we altered energy and stress states of an infrequent feeder, the Terrestrial Gartersnake (*Thamnophis elegans)*, using fasting and repeated restraint stress (Chronic Stressors) to test how these challenges interacted to affect immune function, energy metabolites, and glucocorticoid reactivity (a traditional indicator of stress state) to restraint stress, a standardized, acute stressor. After this acute stressor, the snakes which had received chronic stress had increased glucocorticoid reactivity, and both treatments altered energy metabolite use and storage. Evidence of interaction of food restriction and chronic stress treatments on innate immune function and energy metabolites (triglycerides and glycerol) suggests that stress alters energy use in a manner dependent on the energy state of the animal. Snakes have a remarkable ability to maintain functionality of key physiological processes under stressful conditions but are still susceptible to multiple simultaneous stressors, a situation increasingly prevalent in our ever-changing environment.

INTRODUCTION

Most organisms have a finite amount of energy with which to fuel all of the processes of life (Nagy, Girard & Brown 1999). Most healthy individuals are able to maintain a "dynamic equilibrium" in which energetic expenditure is roughly equal to energetic intake (McCue 2010). Yet often in an individual's life, external or internal perturbations, or stressors, result in a negative energy balance (Wingfield 2005). Energy limitation can cause changes in resource allocation among nonessential processes (French *et al.* 2007; Lucas & French 2012).

Stressors can include predictable changes (e.g., diel shifts and changing seasons) but also unpredictable challenges, such as a loss of food supply (Wingfield 2005; Wingfield 2013). Prolonged food deprivation can be particularly detrimental to an animal for two primary reasons: 1) there is a decrease or cessation in energy input, and/or 2) the physiological response to ongoing food deprivation, a type of chronic stressor, involves the long-term release of energy-mobilizing glucocorticoids (GCs) from the adrenal glands (Chowers, Einat & Feldman 1969; Kitaysky *et al.* 2001; Wingfield & Romero 2001). These hormones are beneficial during an acute stress response but chronic exposure will eventually redirect all available energy from other energetically costly physiological processes, such as reproduction (Vitousek *et al.* 2010), immune function (Lochmiller & Deerenberg 2000), growth (Peterson, Walton & Bennett 1999), and toward protecting the central nervous system (Sapolsky, Romero & Munck 2000). If food deprivation occurs for an extended period of time the organism will experience severe muscle

wasting, increased susceptibility to infection and disease, and ultimately death (Selye 1946; Wingfield 2005).

Because the release of GCs exacerbates the existing shortage of energy during starvation, the level of GCs can provide valuable insight as to how organisms cope with this severe stressor and how organisms shift their limited energy (Romero & Wikelski 2001; Angelier & Wingfield 2013). Glucocorticoids, such as corticosterone (CORT), can be used to assess the unpredictability of food availability in a natural environment (Fokidis *et al.* 2012). However, GC levels alone do not provide adequate representation of the overall health of an organism due to the variable and multifaceted nature of GC secretion (Breuner, Patterson & Hahn 2008; Dickens & Romero 2013). To address this complexity, hypothalamic-pituitary-adrenal axis activity must be examined in conjunction with other physiological processes that directly influence the condition and fitness of the organism, like immunocompetence (Breuner, Patterson & Hahn 2008; Lucas & French 2012; Dickens & Romero 2013).

Immune system efficacy is one of the most important measures in determining the health of an organism (Lochmiller & Deerenberg 2000). It is also highly stress sensitive (Dhabhar *et al.* 2012). During an acute stress event, enhanced immune defense is typically activated by an increase in GCs (Dhabhar & McEwen 1997). The increase in immune components, such as cytokines, causes a further increase in GCs which then acts as negative feedback on the immune system and begins immunosuppression (Dobbs *et al.* 1996; Dhabhar *et al.* 2012). Innate immunity, such as bactericidal ability in the plasma, can provide information about a variety of immune components such as phagocytes, opsonizing proteins, and natural antibodies (French & Neuman-Lee 2012). Measuring the circulating levels of immune cells (bactericidal ability) in

conjunction with examining cutaneous wound healing can provide information on the action of the immune cells that have aggregated at the site of infection (Smith & Barker 1988; French, Matt & Moore 2006; Dhabhar *et al.* 2012; Neuman-Lee & French 2014). In the context of the current study, the high energetic costs associated with immunity make it an ideal metric for energetic prioritization and energy shifts (Lochmiller & Deerenberg 2000).

When undergoing food deprivation, animals must utilize existing resources. Organisms undergoing food deprivation will typically utilize carbohydrates initially, followed by fat stores, and finally will begin to catabolize proteins and nucleic acids (Cherel, Stahl & Le Maho 1987; McCue 2010). Measuring energy metabolites is critical to elucidating the use of these energetic stores (Jenni *et al.* 2000; Seaman, Guglielmo & Williams 2005; McCue 2007b). Concentrations of plasma metabolites, such as triglycerides, glycerol, proteins, and ketones, can indicate energy utilization (McCue 2010; Fokidis *et al.* 2011). These metabolites can indicate chronic stress but also can rapidly change, which allows for the detection of acute stressors (Fokidis *et al.* 2011). As an added benefit in the context of stress physiology, triglycerides are a measure of gluconeogenic activity and therefore can complement the more traditional measurement of glucocorticoid concentrations (Fokidis *et al.* 2011).

Up to now, studies on immune and endocrine consequences of food deprivation and starvation have focused primarily on avian and mammalian models that use entirely different energetic strategies than other vertebrates, such as squamate reptiles (Nakamura *et al.* 1990; Kitaysky *et al.* 2001; Wingfield 2005; Dhabhar *et al.* 2012). Little research has examined ectothermic organisms which regularly undergo energetic limitations and are uniquely adapted to fast for long periods of time, such as many species of snakes (Secor & Diamond 1998). Reptiles

provide an alternative model to examine how stress and energetic state interact to influence an animal's endocrine and immune systems.

Gartersnakes (Genus *Thamnophis,* Reptilia: Colubridae) are an ideal organism for this type of study because of their feeding strategies. Most snakes eat large prey relative to their bodies, but infrequently (Secor & Diamond 1998). To facilitate this strategy, snakes can reduce their already low metabolic rate to accommodate energetic constraints (Hulbert & Else 1981; McCue 2007a). They accomplish this partly by downregulating the energetic costs of maintaining the digestive tract during periods of fasting (Secor & Diamond 2000) through a suite of physiological and morphological changes (Secor, Stein & Diamond 1994; Starck & Beese 2002), allowing snakes to maintain physiological processes even after prolonged periods of fasting (McCue 2007a; McCue 2007b). Even with these adaptations, snakes must still allocate limited resources among multiple costly physiological processes, potentially resulting in energetic trade-offs.

In the present study, we examined the physiological effects of fasting, chronic restraint stress, and their interaction on stress reactivity and innate immune function in Terrestrial Gartersnakes (*Thamnophis elegans*). To provide insight into resource utilization, we measured baseline metabolite concentrations (true triglycerides, glycerol, ketones, and proteins) at the end of a 40-day fasting period (baseline) and then again following a 30-minute acute stress challenge (post-acute challenge). We hypothesized that baseline levels would show differences in energetic metabolites with fasted snakes having lower levels of triglycerides, chronically stressed snakes having higher levels of glycerol, and all groups having similar levels of ketones and proteins. We hypothesized the chronically stressed snakes would have higher levels of corticosterone and

snakes undergoing chronic stress, food restriction, or both would have suppressed bactericidal ability and decreased percent of wound healed. We hypothesized that, relative to baseline levels, following acute stress 1) glycerol concentrations would decrease in chronically stressed snakes and increase in non-chronically stressed snakes, 2) triglycerides and ketones would increase regardless of stress-state or food restriction, and 3) protein concentrations would not change. We predicted that post-acute stress corticosterone levels would be higher in the chronically stressed snakes. Finally, we hypothesized an increase in bactericidal ability in all groups after the acute stress challenge.

MATERIALS AND METHODS

Animal model

We collected 37 male Terrestrial Gartersnakes (*Thamnophis elegans*) from Cache Valley, Utah in September 2011. Only males were used in this study to remove the potential variability of resource allocation to reproduction as females in this population do not reproduce every year (LNL, unpublished data). Snakes were individually overwintered in dark temperature-controlled chambers (5°C) until April 2012 to reduce negative or confounding effects of captivity and then were removed from the chambers and housed individually in 37.8 L glass aquaria with newspaper substrate, a water dish, and a plastic shelter filled with moist peat moss. Heat tape at one end of the aquarium provided a thermal gradient. The room was kept at 26°C on a 12:12 on:off light cycle. All procedures and protocols were approved by the USU IACUC (protocol #2299).

Experimental design

We randomly assigned snakes to four treatment groups: No stress/Food (NS/F, n = 10), No Stress/No Food (NS/NF, n = 10), Chronic Stress/Food (CS/F, n = 8), and Chronic Stress/No Food (CS/NF, n = 9). After randomization, we conducted an analysis of variance on snake body condition (computed as the residual of the regression of snout-vent length (SVL) on mass; Ujvari and Madsen, 2006) comparing the four treatments and showed no difference between groups. There were additionally no differences in SVL or mass ($F_{(3,34)} = 0.57$, p = 0.64 and $F_{(3,34)} = 0.61$, p = 0.61, respectively). Snakes were offered thawed mice twice before food restriction began. Each snake consumed at least one meal prior to starting the food restriction regime. The food restriction protocol was implemented for 40 days. Because snakes being stressed daily (our chronic stress protocol) typically will not eat (LNL, personal observation), chronic stress manipulations were not added until day 29 and continued through day 40 (Figure 1).

The snakes in the Food treatment (NS/F and CS/F) groups were given 20% of their body weight in thawed, previously frozen mice weekly while the snakes in the No Food treatment (NS/NF and CS/NF) groups were fasted (Figure 1). Mice that were not eaten were removed after 24 hrs and recorded. Each snake in the Chronic Stress treatment (CS) groups was removed from its cage daily and placed into an opaque, breathable bag for 30 minutes, after which the snake was replaced in its cage. All snakes in the NS group remained in their aquaria, which were covered while the researchers were present to ensure that human presence did not cause a stress response. All snakes were weighed weekly when cages were cleaned.
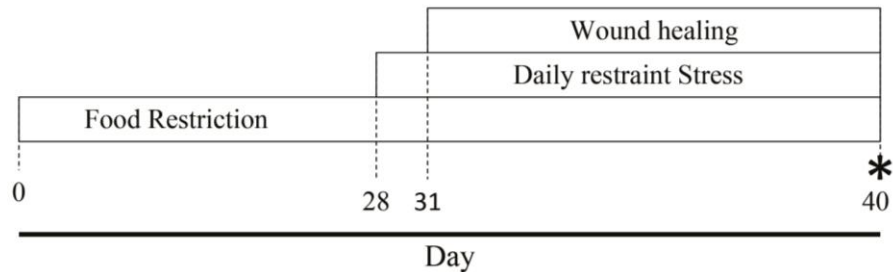
Fig. 8-1. Timeline of experimental procedures testing the effects of food restriction and chronic stress on stress reactivity, immune function, and plasma metabolite levels in male gartersnakes (*Thamnophis elegans*). The asterisk on day 40 indicates the stress reactivity test (baseline and post-acute stress samples).

Acute stress challenge

To obtain baseline samples of (1) free glycerol, (2) total triglycerides, (3) the ketone, β-hydroxybutyrate, (4) total protein (5) CORT and (6) bactericidal ability, all snakes were removed from their cages on the final day of the experiment (Day 40) and within a minute a blood sample was taken from the caudal vein (Romero & Reed 2005). The snake was then placed in a separate, opaque, breathable bag for 30 minutes after which the snakes were removed and a second blood sample, post-acute stress sample, was collected (Figure 1). All blood sampling was completed between 0900 and 1100. The samples were kept on ice until all samples were collected and then centrifuged at 2200 rpm for 10 minutes. The plasma was separated and frozen at -20°C until further analysis.

Metabolite assays

To assess how stress and food deprivation alter the usage of energy fuels from different physiological stores, we measured plasma levels of the four metabolites listed above in both baseline and post-acute stress samples: 1) free glycerol, indicating the breakdown of liver and adipose triglyceride stores; 2) total triglycerides, the circulating levels of both free glycerol and triglycerides; 3) the ketone, β-hydroxybutyrate, which correlates with the oxidation of fatty acids (a component of triglycerides), and 4) total protein, a measure of overall circulating albumin and globulins, indicative of starvation and muscle wasting.

Free glycerol was measured using a spectrometric endpoint assay, and then on the same assay for the same samples total triglycerides were measured (for details, see Guglielmo *et al.* 2002, Fokidis *et al.* 2011, and Fokidis *et al.* 2012). The 'true' triglyceride concentration was defined as the difference between the total triglycerides and free glycerol concentrations and indicates deposition into adipose tissue. Plasma concentrations of ketones and proteins were determined using a commercial colorimetric assay (#700190, Cayman Chemical Co., Ann Arbor, MI, USA) and a Bradford assay modified for plasma (see Bradford 1976; Okutucu *et al.* 2007), respectively. Assay sensitivities were 0.16 mM (glycerol and triglycerides), 0.01 mM (ketones) and 0.93 g/dl (proteins). The mean intra- and inter-assay coefficients of variation were: 5.9 and 10.1% for glycerol; 6.7 and 9.8% for triglycerides; 7.8 and 16.4% for ketones, and 3.8 and 6.9% for proteins. Validations included tests for parallelism between a standard curve and serially-diluted samples derived from pooled gartersnake plasma (all $p > 0.069$) and recovery of spiked samples ($79 \pm 11.4\%$, $82\% \pm 16.1$, $88\% \pm 7.4$, and $94\% \pm 10.7$ for glycerol, triglycerides, ketones, and proteins, respectively).

Radioimmunoassay for corticosterone

Circulating corticosterone concentrations in both baseline and post-acute stress samples were determined using a radioimmunoassay protocol modified from French *et al.* (2008). Samples were extracted using a solution of 30% ethyl acetate:isooctane in duplicate for CORT (MP Biomedicals, Lot #3R3PB-19E). Final concentrations were calculated by averaging the duplicate samples and adjusted using individual recoveries (average 76.1%). Intra-assay variation was 11.3% and precision was 90.9%.

Bactericidal assay

We performed the bactericidal assay in baseline and post-acute stress samples following the protocol outlined in French and Neuman-Lee (2012). Briefly, we combined a 1:5 plasma dilution with CO_2-Independent media plus 4 nM L-glutamine, 10^4 colony producing units *Escherichia coli* (EPowerTM Microorganisms #483-237-1, ATCC 8739, MicroBioLogics, St. Cloud, MN, USA), and agar broth on a 96-well microplate. We incubated the plate for 12 h and calculated absorbance using a microplate reader (300 nm, BioRad Benchmark, Hercules, CA, USA).

Cutaneous biopsy

On day 31, three days after initiation of chronic stress treatment, each snake was given a cutaneous biopsy on its dorsal surface using a 3.5 mm biopsy punch (Miltex Instruments, York, PA, USA). Based on a previously validated protocol (French, Matt & Moore 2006), we waited three days to ensure that the snakes were in the experimental stress state, given that the initial

healing period is so critical. The punch was lightly twisted and the circular portion of skin

removed using forceps. All wounds were photographed immediately after being administered.

After securing the snake with Velcro restraints, a ruler was placed in the same plane as the

wound for scale, and the wound was photographed with a digital camera. At the termination of

the experiment (Day 40), another image was taken in the same manner. Previous studies in

reptiles found that nine days was adequate to detect stress-related differences in wound healing

(French, Matt & Moore 2006, Neuman-Lee & French 2014). Images were analyzed using ImageJ

v. 1.48 (National Institute of Mental Health, Bethesda, MD, USA) to estimate wound area.

Wound measurements were blinded such that the investigator did not know treatment

assignments.

Statistical analyses

To assess the effects of chronic stress and fasting treatments on change in body mass over

the course of the experiment, we used a two-way factorial in a completely randomized design

with repeated measures in a generalized linear mixed model. The effects of chronic stress and

fasting treatments on baseline values of each plasma measure, bactericidal ability, and wound

healing were assessed using a two-way factorial in a completely randomized design, both with

and without initial body condition incorporated as a covariate, using a generalized linear model.

Pairwise comparisons of means among the four treatment combinations were adjusted for

inflated Type I error using the Tukey-Kramer method. To evaluate the impact of the acute stress

challenge on each plasma measure and bactericidal ability, we used a two-way factorial in a

completely randomized design with repeated measures, both with and without body condition

incorporated as a covariate, using a generalized linear mixed model. For each response, we omitted snakes for which we did not have both baseline and post-acute stress data. Tests of acute stress challenge effect (i.e., the difference between post-stress and baseline values) for all fasting and stress treatment combinations were adjusted using the stepdown Bonferroni method. Pairwise comparisons of acute stress challenge effects among the four treatment combinations also were adjusted using the stepdown Bonferroni method. Bactericidal ability and wound healing (measured as the proportion of initial wound area minus final wound area to initial wound diameter) were analyzed using a beta distribution with a logit link; protein, free glycerides, ketone, true triglycerides, and corticosterone were analyzed using a normal distribution following transformation ($\log_e$ for mass, protein, and glycerol; square-root for ketone, triglyceride, and corticosterone) to better meet assumptions of normality and homogeneity of variance. Two baseline observations were omitted from the analysis of protein due to unrealistic values that could not be resolved. Body condition was not a significant factor in any analysis ($P > 0.139$), thus we report here models without body condition. The baseline bactericidal ability between the NS/F and CS/NF group was analyzed using a Kruskal-Wallis test.

All analyses were performed using the GLIMMIX procedure in SAS/STAT 13.2 in the SAS System for Windows 9.4 (SAS Institute Inc., 2014), with the exception of the initial body mass and SVL analyses and the Kruskal-Wallis test, which was conducted on JMP 11.0 (SAS Institute Inc., 2013).

RESULTS

Body mass and energy metabolites

Fed (F) snakes gained more mass than nonfed (NF) snakes, and CS snakes gained less mass than NS snakes (Tables 1a and 1b; Figure 2). There is some evidence that the effect of food on mass increase was enhanced in NS snakes.

Both fasting and chronic stress increased glycerol baseline concentrations. Relative to baseline concentrations, post-acute stress concentrations decreased for snakes experiencing chronic stress and increased for snakes experiencing no chronic stress (Tables 1a and 1b and 2, Figure 3A).
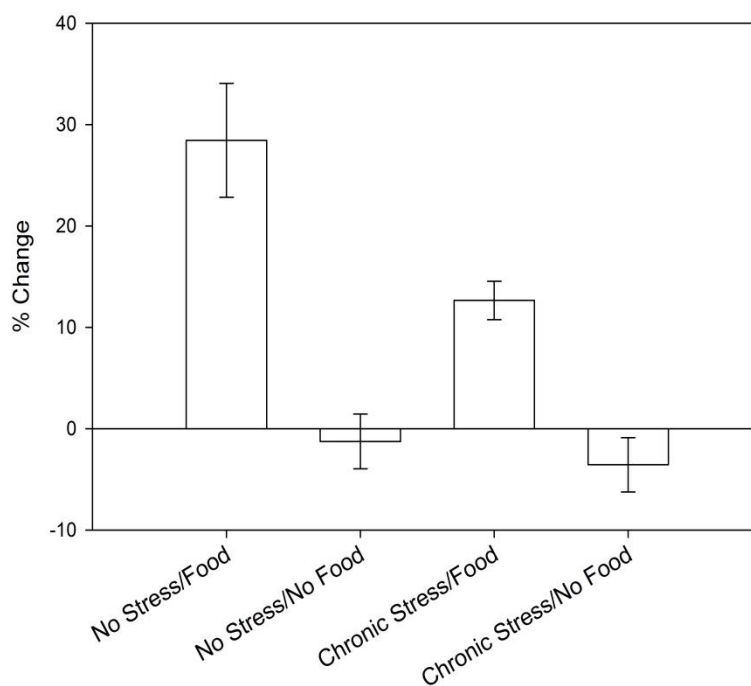
Fig. 8-2. Mass change in male gartersnakes (*Thamnophis elegans*) as a consequence of treatment with food restriction, chronic stress, neither or both. Bars indicate mean ± 1 standard error computed as descriptive statistics based on the raw data.
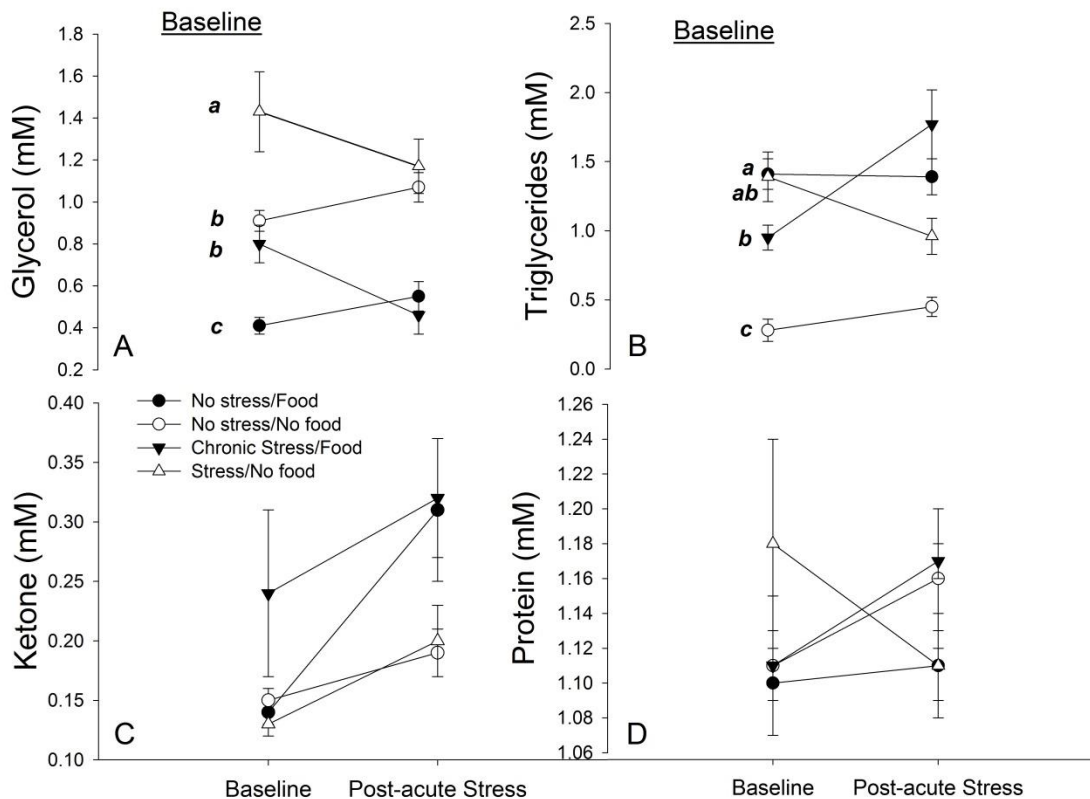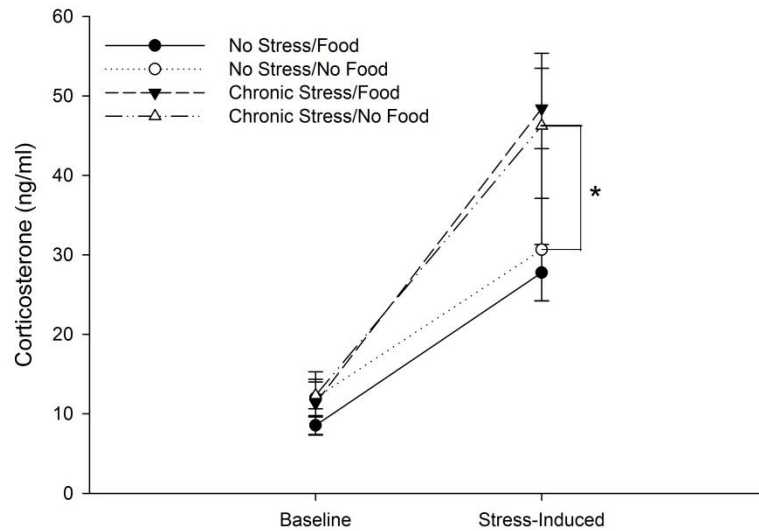
Fig. 8-3. Mean (± 1 standard error) metabolite levels at baseline and following acute stress in male gartersnakes (*Thamnophis elegans*) under food restriction, chronic stress, neither or both: A) glycerol, B) true triglycerides, C) ketone, and D) protein. Values are computed as descriptive statistics based on the raw data. In panels A and B, different letters (lower case) indicate significant differences at baseline.

The true triglyceride baseline concentration was influenced by the interaction of the fasting and chronic stress treatments (Tables 1a and 1b). The NS/NF group had lower concentrations of true triglycerides than the other three groups; the CS/F had intermediate concentrations; and the NS/F and CS/NF groups had highest concentrations (Table 2, Figure 3B).

The difference in concentrations between baseline and post-acute stress samples was affected by the interaction of fasting and stress treatments (Tables 1a and 1b). Relative to the baseline, the post-acute stress concentrations increased for the NS/NF and CS/F groups, decreased for the CS/NF group, and had no apparent change for the NS/F group (Figure 3B).

There was no evidence of a fasting or chronic stress effect on baseline concentrations of ketone (Tables 1a and 1b and 2, Figure 3C). Relative to baseline concentrations, post-acute stress concentrations increased in fed snakes.

Protein baseline concentrations were not significantly affected by fasting or chronic stress (Tables 1a and 1b and 2, Figure 3D). Although analysis suggested that the response to the acute stress challenge was affected by the interaction of fasting and chronic stress, post hoc analyses indicated that this result merely reflected noise in the data and was not biologically meaningful.

Stress reactivity

Corticosterone baseline concentrations were slightly higher in fed snakes and there was no evidence of any effect of chronic stress (Tables 1a and 1b and 3, Figure 4). Relative to baseline concentrations, post-acute stress concentrations increased, and the increase was greater for snakes that were chronically stressed.

Fig. 8-4. Mean (± 1 standard error) corticosterone concentrations at baseline and following acute stress in male gartersnakes (*Thamnophis elegans*) under food restriction, chronic stress, neither or both. Values are computed as descriptive statistics based on the raw data. The asterisk indicates a significant difference.

Immune function

Baseline bactericidal ability did not differ among fasting or chronic stress regimes, however the analysis between the NS/F and CS/NF revealed a higher bactericidal ability in the NS/F group (Tables 1a and 1b and 3; Figure 5). The response to the acute stress challenge was affected by the interaction of fasting and chronic stress. There was no evidence of a change from baseline to post-acute stress for any treatment group other than CS/NF, for which bactericidal ability increased. Relative to baseline concentrations, the increase in bactericidal ability in unfed snakes under chronic stress was shown to be different than the change in fed snakes under chronic stress (Tables 1a and 1b and 3; Figure 6). The proportion of wound healed was greater for non-stressed snakes (Tables 1a and 1b, Figure 7).
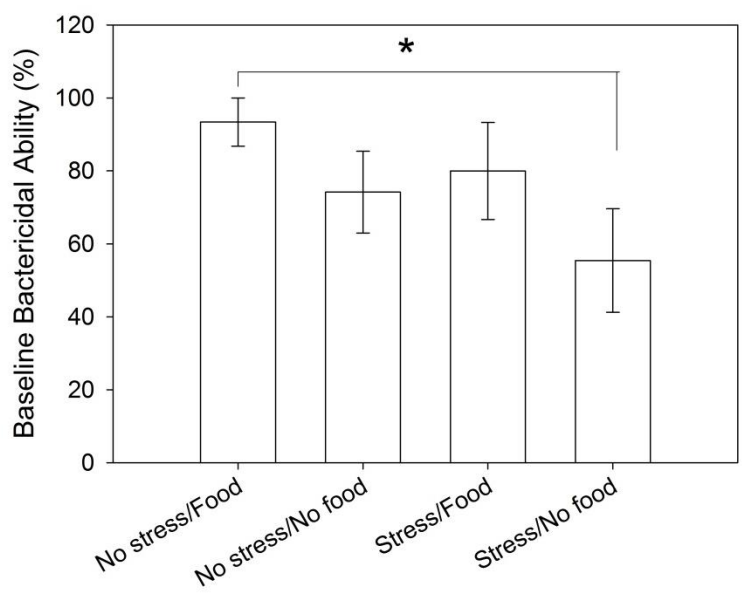
Fig. 8-5. Mean (± 1 standard error) bactericidal ability at baseline in male gartersnakes (*Thamnophis elegans*) under food restriction, chronic stress, neither or both. Values are computed as descriptive statistics based on the raw data. Asterisk indicates that the two groups are significantly different (p = 0.026).
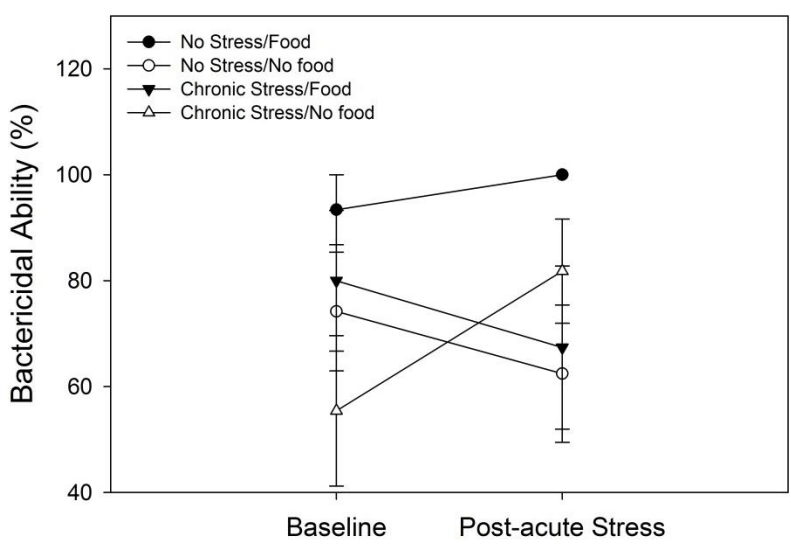
Fig. 8-6. Mean (± 1 standard error) bactericidal ability at baseline and following acute stress in male gartersnakes (*Thamnophis elegans*) under food restriction, chronic stress, neither or both. Values are computed as descriptive statistics based on the raw data.
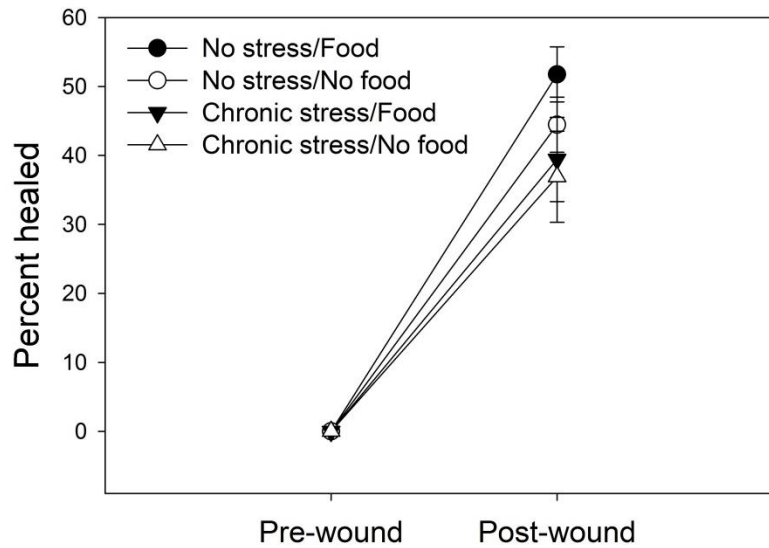
Fig. 8-7. Mean (± 1 standard error) percent area of the cutaneous biopsy healed after nine days in gartersnake (*Thamnophis elegans*) males under food restriction, chronic stress, neither or both. Values are computed as descriptive statistics based on the raw data.

DISCUSSION

Overall, the results of our study indicate that chronic stress and food restriction play important roles in how an organism responds to acute stress. Even in snakes, which are infrequent feeders, food restriction had important physiological consequences. The metabolite levels in snakes in the food restricted groups indicated that they were not undergoing severe energetic limitation because they had not started to utilize protein stores. NF snakes also had not lost substantial body mass, but F snakes had significantly higher increases in body mass. Because snakes can reduce the amount of energy they invest in maintaining their gut (Secor, Stein &

Diamond 1994), it is likely that these individuals had enough energy to shift to other processes, such as their immune system. However, snakes that were chronically stressed were likely secreting more CORT continuously over the treatment period (each time they were removed from their enclosure and stressed) and were therefore more energetically-compromised and could not invest as much in their immune function, resulting in suppressed immunity. The dramatic increase in baseline glycerol in chronically stressed snakes compared to the non-stressed, fed snakes provides strong evidence of gluconeogenesis.

Glycerol, which is indicative of the utilization of adipose stores, is a key substrate for gluconeogenesis (Guglielmo, Cerasale & Eldermire 2005). Snakes that were chronically stressed and fasted had the highest levels of baseline and post-acute challenge glycerol, which corroborates our hypothesis that more energy was mobilized in snakes that were likely experiencing higher levels of corticosterone on a consistent basis. It also demonstrates that snakes were energetically impacted by the 40-day food restriction, even if there was not a dramatic decrease in body mass. The chronically stressed snakes had decreased total glycerol after the acute stress, while the non-chronically stressed snakes had increased total glycerol. An increase in glycerol after acute stress also has been observed in mammals (Ricart-Jane *et al*. 2002) due to the fact that catecholamines (*e.g.,* norepinephrine) stimulate glycerol release (Lin, 1977). However, animals that are chronically stressed show decreased turnover of catecholamines (Konarska, Stewart & McCarty 1989; Roth, Mefford & Barchas 1982) and plasma glycerol concentrations (Fokidis *et al.* 2011). In our study, the chronically stressed snakes may have had a dampened sympathetic-adrenal medullary response, which caused less mobilization of adipose stores, thus decreasing total glycerol concentration.

Triglyceride levels, which represent the amount of glucose being processed by the liver to be stored as adipose (Fokidis *et al.* 2011), were lower in the fasted, non-stressed snakes at baseline and were reduced in the post-acute stress sample in chronically stressed, non-fed snakes. This reduction in triglyceride levels was likely due to the substantial increase in gluconeogenesis during chronic stress over the 12-day period added to the lack of new energetic resources to mobilize for glucose storage. Conversely, the CS/F snakes increased triglyceride levels significantly after the acute stress challenge. NS/NF snakes had slightly higher levels. An increase in triglycerides after acute stress has been documented (Stoney *et al*., 1988) but contradicts other studies showing that acute stress, regardless of previous stress state, causes a decrease in triglyceride levels (Ricart-Jane *et al*. 2002; Teague *et al*., 2007).

Baseline ketone levels did not differ among treatment groups. We expected to see higher ketone levels in the fasted snakes (Akram 2013). However, given snakes' ability to fast for long periods of time, a six-week treatment period may not have been long enough. For all treatments, ketone levels increased after the acute stress challenge. This increase after an acute stress is consistent with previous studies conducted with rats (Ricart-Jane *et al*. 2002; Teague *et al*., 2007).

At baseline, protein levels were not different among the treatment groups, nor was there any consistent evidence of effects of fasting or chronic stress on response to acute stress. This provides evidence that snakes, like most animals, preferentially preserve protein for energy during later stages of starvation, which were not reached (Cherel, Stahl & Le Maho 1987; McCue 2010).

In this study, the total CORT released after the acute stress challenge was higher in snakes which had been chronically stressed. While this increase in CORT levels after a stress challenge is consistent (though not universal) with previous reptile studies (reviewed in Moore & Jessop 2003), it also is consistent with findings that the amount of change can vary widely across species, populations, and individuals depending upon context (Dickens & Romero 2013). Clearly, an animal will respond differently depending upon recent stressful events and whether or not it has eaten. Therefore, it is critical to evaluate the CORT response in context of the organism's natural history.

Despite the effects on stress reactivity, there was no evidence that chronic stress affected baseline CORT, and only little evidence that food restriction elevated CORT, which is a traditional stress indicator. Our results demonstrate the need to exercise caution when interpreting baseline CORT levels, because chronic stress treatment did not influence baseline CORT as predicted. Much work has correlated fitness or health of a population to baseline CORT levels across many taxa (Hopkins, Mendonça & Congdon 1997; Marra & Holberton 1998; Romero & Wikelski 2001; Jaatinen *et al.* 2013), although there is strong evidence that fitness and CORT do not necessarily have a predictable relationship (Breuner, Patterson & Hahn 2008; Bonier *et al.* 2009). Our work reinforces the need to examine stress-responsiveness in addition to other fitness related metrics to more fully determine stress status and its consequences for the organism (Breuner, Delehanty & Boonstra 2013).

There is not a clear consensus in the literature as to what constitutes an "optimal stress response" (Dickens & Romero 2013), requiring additional measures (Rich & Romero 2005; Lattin *et al.* 2012; Brooks & Mateo 2013; Jessop, Woodford & Symonds 2013). Corticosterone

is a mediator for a wide variety of physiological processes, including the immune response (Dhabhar *et al.* 1995; Dhabhar *et al.* 1996). This relationship between CORT and immune activity can allow us to link the stress-response with a functional measure of energetic investment in self-maintenance. For example, acute stress typically corresponds with an increase in immune function when energy is available (Dhabhar 2009) and the individuals in the NS/F treatment in the present study had the most effective bactericidal ability that was further augmented after the post-acute stress increase in CORT, which is consistent with previous studies (Dhabhar & McEwen 1997; Dhabhar 2002). Chronically elevated levels of CORT typically correspond with a suppression of immune processes (Dhabhar & McEwen 1997). Here we find that animals with both chronic stress and food restriction have suppressed bacterial killing response relative to the NS/F animals (akin to controls). Additionally, we believe the fact that all but one NS/F snakes had 100% bactericidal killing relative to the assay control for baseline and then all NS/F snakes had 100% killing after the acute stress challenge is biologically significant. In further support of this theory, there was a significant interaction of food, stress, and time, with snakes experiencing either food restriction, chronic stress, or the combination of responding differently via bactericidal ability following an acute stress challenge.

We found consistent results with our other measure of immunity, wound healing. In the present study, snakes undergoing chronic stress had reduced healing ability relative to the non-stressed snakes. Wound healing has been shown to be negatively affected by chronic stress due to the suppression of proinflammatory cytokines (Christian *et al.* 2007) and increased CORT levels (Padgett, Marucha & Sheridan 1998; French, Matt & Moore 2006).

CONCLUSIONS

This study demonstrates that energetic state, in combination with energy mobilizing GCs, can affect vital processes for health and survival. Thus, this evidence suggests that stress alters energy use and immunity in a manner dependent on the energy state of the animal. Although an acute stress challenge altered stress reactivity, chronic stress treatment increased reactivity and did not affect baseline CORT concentrations. Baseline glycerol, an indicator of gluconeogenesis, was increased in all snakes, even in the absence of altered baseline CORT. This suggests that hormonal indicators may not detect all important consequences of stress. This work also demonstrates the resilience of snakes in dealing with both stressors and infrequent food supply. These snakes have a remarkable ability to maintain functionality of key physiological processes under stressful conditions but are still susceptible to multiple simultaneous stressors. The combined effects of fasting and repeated stress are probably not far removed from the myriad stressors these snakes encounter under natural conditions.

Table 1a. Analysis of results at baseline. Significant findings are denoted with an asterisk.

	Degrees of Freedom	Food	Stress	Food* Stress	Time	Food*Time	Stress*Time	Food*Stress*Time
Baseline								
Triglycerides	$F_{(1,33)}$	15.30	13.59	48.27	-	-	-	-
	P	<0.001*	<0.001*	<0.001*				
Glycerol	$F_{(1,33)}$	32.76	19.29	1.44	-	-	-	-
	P	<0.001*	<0.001*	0.239				
Ketones	$F_{(1,33)}$	1.52	0.67	2.47	-	-	-	-
	P	0.226	0.419	0.125				
Protein	$F_{(1,31)}$	0.86	0.71	0.59	-	-	-	-
	P	0.361	0.406	0.447				
Corticosterone	$F_{(1,31)}$	3.33	0.01	0.35	-	-	-	-
	P	0.078	0.926	0.557				
Bactericidal Ability	$F_{(1,33)}$	1.50	1.38	0.00	-	-	-	-
	P	0.229	0.249	0.949				
Wound Healing	$F_{(1,33)}$	1.76	4.94	0.01				
	P	0.194	0.033*	0.938				
Mass	$F_{(1,33)}$	2.16	0.69	0.36	15.55	26.16	5.21	3.20
	P	0.151	0.413	0.552	<0.001	<0.001	0.029	0.083

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Table 1b. Analysis of results after the acute stress challenge. Significant findings are denoted with an asterisk.

Acute Stress Challenge	Degrees of Freedom	Food	Stress	Food*Stress	Time	Food*Time	Stress*Time	Food*Stress*Time
Triglycerides	$F_{(1, 33)}$	38.64	17.71	24.75	3.11	4.14	0.05	17.87
	P	< 0.001	< 0.001	< 0.001	0.087	0.050*	0.831	< 0.001*
Glycerol	$F_{(1, 33)}$	64.05	4.63	0.00	1.53	1.60	13.23	3.02
	P	< 0.001	0.039	0.956	0.224	0.215	< 0.001*	0.092
Ketones	$F_{(1, 33)}$	5.55	0.39	0.67	31.30	4.18	0.20	2.50
	P	0.025	0.538	0.417	< 0.001	0.049*	0.657	0.123
Protein	$F_{(1, 31)}$	0.24	0.44	0.28	0.65	1.15	0.43	5.22
	P	0.628	0.514	0.602	0.427	0.291	0.518	0.029*
Corticosterone	$F_{(1, 26)}$	0.14	6.93	0.11	143.99	1.72	8.55	1.41
	P	0.714	0.014	0.747	< 0.001	0.201	0.007*	0.245
Bactericidal Ability	$F_{(1, 30)}$	3.43	3.05	3.18	2.31	0.38	0.08	6.02
	P	0.074	0.091	0.085	0.139	0.541	0.784	0.020*

Table 2. Metabolite concentrations in baseline and post-acute stress plasma samples taken from male gartersnakes (*Thamnophis elegans*) under food restriction, chronic stress, neither or both. Delta values are the mean differences between baseline and post-acute stress measurements. Values are means ± 1 standard error computed as descriptive statistics based on the raw data.

Treatment	Baseline				Post-acute stress				Delta			
	Glycerol (mM)	True Triglyceride (mM)	Ketone (mM)	Protein (g/dL)	Glycerol (mM)	True Triglyceride (mM)	Ketone (mM)	Protein (g/dL)	Glycerol (mM)	True Triglyceride (mM)	Ketone (mM)	Protein (g/dL)
No stress/Food (NS/F) n = 10	0.41 ± 0.04	1.41 ± 0.11	0.14 ± 0.01	1.10 ± 0.03	0.55 ± 0.07	1.39 ± 0.13	0.31 ± 0.06	1.11 ± 0.02	0.14 ± 0.09	0.0 ± 0.18	0.17 ± 0.05	0.004± 0.02
No stress/No food (NS/NF) n = 10	0.91 ± 0.05	0.28 ± 0.08	0.15 ± 0.01	1.11 ± 0.04	1.07 ± 0.07	0.45 ± 0.07	0.19 ± 0.02	1.16 ± 0.04	0.16 ± 0.07	0.17 ± 0.10	0.04 ± 0.02	0.05 ± 0.05
Chronic stress/Food (CS/F) n = 8	0.80 ± 0.09	0.95 ± 0.09	0.24 ± 0.07	1.11 ± 0.02	0.46 ± 0.09	1.77 ± 0.25	0.32 ± 0.05	1.17 ± 0.01	-0.3 ± 0.15	0.82 ± 0.25	0.08 ± 0.04	0.06 ± 0.02
Chronic Stress/No food (CS/NF) n = 9	1.43 ± 0.19	1.39 ± 0.18	0.13 ± 0.01	1.18 ± 0.06 (n = 7)	1.17 ± 0.13	0.96 ± 0.13	0.20 ± 0.03	1.11 ± 0.03 (n = 7)	-0.26 ± 0.11	-0.4 ± 0.12	0.07 ± 0.03	-0.07± 0.06 (n = 7)

Table 3. Physiological measurements in male gartersnakes (*Thamnophis elegans*) under food restriction, chronic stress, neither or both. Values are means ± 1 standard error computed as descriptive statistics based on the raw data.

Treatment	Baseline		Post-acute Stress		Delta		
	CORT (ng/ml)	Bactericidal ability (%)	CORT (ng/ml)	Bactericidal ability (%)	CORT (ng/ml)	Bactericidal ability	% Wound Healed
No stress/Food (NS/F) n = 10	8.54 ± 1.2 (n = 9)	93.40 ± 6.6	27.77 ± 3.5 (n = 9)	100 ± 0 (n = 9)	19.23 ± 3.4 (n = 9)	7.34 ± 7.3 (n = 9)	51.74 ± 4.0
No stress/No food (NS/NF) n = 10 Chronic	11.99 ± 2.4	74.18 ± 11.2	30.65 ± 6.5 (n = 9)	62.44 ± 13.0 (n = 9)	19.65 ± 6.1 (n = 9)	-8.86 ± 4.4 (n = 9)	44.45 ± 4.0
Chronic stress/Food (CS/F) n = 8 Chronic	8.08 ± 2.5 (n = 7)	74.97 ± 13.3	53.0 ± 5.46 (n = 5)	67.38 ± 15.4 (n = 7)	43.95 ± 5.2 (n = 5)	-4.02 ± 12.5 (n = 7)	39.42 ± 6.1
Stress/No food (CS/NF) n = 9	12.32 ± 1.7	55.42 ± 14.2	46.25 ± 9.1 (n = 7)	81.79 ± 9.84	33.62 ± 8.0 (n = 7)	26.38 ± 17.8	36.91 ± 6.6

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CHAPTER 9

CONCLUSION

The goal of my research was to provide a wide spectrum of studies to analyze the

potential responses of herpetofauna to various forms of stress. My research took many

forms, examining behavior, natural toxins, food restriction, and anthropogenic toxins.

The disparity between the stressors and model organisms can make direct comparisons

difficult, yet the overall conclusions of these studies mirror those of most other research

on stress: We know very little and must endeavor to learn more about physiology in the

context of stress.

Context-dependency

As the field of ecophysiology has grown, we are becoming more aware that many

relationships that have been reliably measured in the lab are not mirrored in the field

(Calisi & Bentley 2009; Marra et al. 1995). Because so much theory has been based on

findings in laboratory settings, it can be difficult to understand why we fail to see

repeated relationships and, in some cases, see relationships that work in the opposite

directions across the taxonomic spectrum (Baker et al. 2013; Dickens & Romero 2013;

Goessling et al. 2015; Jessop et al. 2013; Moore & Jessop 2003).

These variations have shown that the stress response is extremely context-

dependent (Baugh et al. 2014; MacDougall-Shackleton et al. 2013; McCormick et al.

2015). What may cause a major stress response in one organism may not elicit a response

at all in another. This is exemplified in my findings shown in Chapter 5. Female snakes responded strongly to TTX when we measured their CORT levels. However, male and juvenile snakes had no measurable response. This dynamic represents the complication of examining such a context-dependent process. Animals may change their stress response (and thus all downstream effects from reproduction to immune function) based on a wide variety of factors.

For example, life-history is an important factor in determining the energetic investment into the stress response, immune response, reproduction, and other processes (Glennemeier & Denver 2002; Ketterson & Nolan 1992; Lucas & French 2012; Palacios et al. 2012). Longer lived organisms may invest more in their immune response than reproduction given that they will have more reproductive periods (Stearns 1989). Given that glucocorticoids (GCs) are often considered mediators between these different physiological investments, levels of GCs may shift accordingly (Love et al. 2004; Palacios et al. 2012; Vitousek et al. 2010; Zera & Harshman 2001). Other factors that have been shown to impact stress physiology are: reproductive status (French et al. 2007; Moore et al. 2000; Salvante & Williams 2003; Taylor et al. 2004), time of day (Breuner et al. 1999; Tarlow et al. 2003), energetic status (Fokidis et al. 2011; Holberton 1999; Kitaysky et al. 2001; Schoech et al. 2007), genetic make-up (Evans et al. 2006), life-history stage (Landys et al. 2006; Lattin et al. 2012), time of year (Holberton 1999; Marra & Holberton 1998), infection status (Gabor et al. 2013), and natal environment (Crino et al. 2014; DuRant et al. 2010; Haussmann et al. 2012; Love & Williams 2008). Even within the same population, there are high amounts of variability among individuals

(Baugh et al. 2014; Jaatinen et al. 2013; Wada et al. 2008) when measuring their stress and immune physiology. These layers of complexity make it very difficult to accurately measure the overall patterns within a population or species.

In analyzing my research findings, I was unable to determine a reliable pattern between natural or anthropogenic stress (Table 9-1). While some natural stressors elicited an increase in CORT (Chapter 3, wounding in lizards; Chapter 5, TTX in female snakes), others did not (Chapter 4, antipredator behavior in newts; Chapter 5, TTX in male and juvenile snakes; Chapter 8, food restriction in snakes). Similarly, some anthropogenic stressors were associated with an increase in CORT (Chapter 6, INDOX exposure in snakes; Chapter 8, handling in snakes), while others were not (Chapter 7, BDE-47 in snakes). This lack of consistency also continued when analyzing bactericidal ability (Table 9-1). I saw a decrease in bactericidal ability in one study with a natural stressor (Chapter 3) and no effect with the other studies and natural stressors. Conversely, I measured an increase in bactericidal ability when snakes were challenged with INDOX (Chapter 6). Finally, when both a natural and anthropogenic stressor were applied (Chapter 8), there was a decrease in bactericidal ability.

Because there is so much evidence showing strong differences in responses between chronic and acute stressors (Moore et al. 1991; Teague et al. 2007; Wingfield & Romero 2001), I also analyzed my findings through that lens. Similarly to anthropogenic vs. natural stressors, I found no consistent pattern (Table 9-1).

Table 9-1. Compiled findings from research on the physiological responses in herpetofauna to different types and durations of stressors.

Study	Stressor	Duration	Stress Response	Immune Response	Reproductive Response
Wound healing in side-blotch lizards	Natural Wounding	Chronic	Increased CORT level	Decreased bactericidal ability	Not measured
Antipredator behavior in newts	Natural Predator attack	Acute	Increased CORT level	Not measured	Not measured
Response to TTX in gartersnakes	Natural Natural toxin	Chronic	Increased CORT in females only	No effect	Not measured
Response to TTX and Indoxacarb in gartersnakes	Natural/ Anthropogenic Toxin	Acute	Increased CORT to INDOX only	Increased bactericidal ability with INDOX only	No change in testosterone
Effects of BDE-47 on pregnant gartersnakes	Anthropogenic Toxin	Chronic	No effect	No effect	Possible reduction in offspring viability
Food restriction and chronic stress in gartersnakes	Natural/ Anthropogenic Food Restriction Handling	Chronic	No effect on baseline; Increased reactivity when chronically stressed	Decreased bactericidal ability when challenged with both stressors	Not measured

Given the inherent variability, it may seem that measuring the stress response is a futile endeavor. However, when combined with related physiological, ecological, or behavioral metrics (upstream or downstream), the stress response can be a valuable indicator of individual health (Cabezas et al. 2007; Marra & Holberton 1998; Romero & Wikelski 2001; Walker et al. 2005). Because of this, I was careful to measure at least one other variable in my studies. This is primarily because the variability in GC levels can render the data virtually meaningless without further metrics for interpretation. There has been rigorous debate on what to measure in conjunction with total GC levels, but most stress physiologists agree that at least measuring immune and reproduction are key to understanding how GC levels affect fitness (Breuner et al. 2013; Schoech et al. 2013).

Herpetofauna

My work has added a significant portion to the sparse knowledge of stress physiology in herpetofauna. While various studies have examined the effect of some parameter on CORT only (Gabor et al. 2013; Tempel & Gutiérrez 2004; Walker et al. 2005), most studies have not examined multiple levels of physiology or behavior, although there are clearly exceptions (Deviche et al. 2014; French et al. 2008; French et al. 2010; Refsnider et al. 2015). My dissertation has also added greatly to the reptile ecotoxicology literature. Reptiles are probably the least studied taxon in terms of ecotoxicology (Hopkins 2000; Weir et al. 2010), potentially due to the Environmental Protection Agency considering bird models an adequate proxy for reptile responses (Weir et al. 2010). While this may be appropriate for some chemicals, it is clearly not for all (or

even most) chemicals, especially chemicals that are easily absorbed across the skin (Weir et al. 2010; Weir et al. 2014; Weir et al. 2015). Additionally, reptiles and amphibians generally have lower metabolic rates than birds (Andrews and Pough 1985).

While my research on ecotoxicology on snakes has been important, it has also followed the trend of many ecotoxicological studies in reptiles: Reptiles are very resilient. In many studies, little to no response has been found to a wide variety of chemicals (de Solla et al. 1998; de Solla et al. 2006; Ganser et al. 2003; Neuman-Lee & Janzen 2011; Neuman-Lee et al. 2014; Weir et al. 2010). These same chemicals often elicit a highly significant response in amphibians (Hayes et al. 2010; Hopkins et al. 1997; Relyea 2004; Rohr et al. 2003; Rohr et al. 2006). This is likely why it is difficult to work in the field of reptilian ecotoxicology. Many studies with negative data have not been published in this field (R. Parker, personal communication; S. Weir, personal communication, L. Neuman-Lee unpublished data). This publishing bias is very real and may be hurting the field.

Conclusions

With the increased risks to herpetofauna, including *Bactrachochytrium dendrobatidis* and *B. salamandrivorans*, snake fungal disease, habitat destruction, climate change, and pollutants, we may see further declines (Gibbons et al. 2000; Martel et al. 2014; Sutherland et al. 2014; Visser 2008). While these organisms have inhabited the earth for millions of years, the multiple anthropogenic pressures may force these resilient organisms to either adapt rapidly or face extinction.

My research provides a greater perspective on how some reptiles and amphibians modulate their physiological responses, especially with respect to stress and immune physiology. By providing a wide spectrum of stressor types, I was able to confirm that many of these responses are highly variable and context-dependent; thus each situation must be evaluated individually before policy or management decisions can be made. While this may prove to be more time and resource intensive, it is clearly the most effective method when evaluating physiological responses.

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APPENDICES

Appendix A: Coauthor Permission Letters

January 25, 2016

Ms. Lorin A. Neuman-Lee
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Lorin,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Neuman-Lee LA, Stokes AN, Greenfield S*, Hopkins G, Brodie ED, Jr., French SS. 2015. Effects of antipredator behavior and tetrodotoxin concentrations on stress response in Rough-skinned newts. *General and Comparative Biology*. 213:59-64.

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Amber Stokes
Assistant Professor
Department of Biology
California State University at Bakersfield

January, 26th 2016

Ms. Lorin A. Neuman-Lee
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Lorin,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Neuman-Lee LA, Stokes AN, Greenfield S*, Hopkins G, Brodie ED, Jr., French SS. 2015. Effects of antipredator behavior and tetrodotoxin concentrations on stress response in Rough-skinned newts. *General and Comparative Biology*. 213:59-64.

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Sydney Greenfield
Master's Student
Wildlife Institute, School of Nature Conservation
Beijing Forestry University

January 25, 2016

Ms. Lorin A. Neuman-Lee
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Lorin,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Neuman-Lee LA, Stokes AN, Greenfield S, Hopkins GR, Brodie, Jr. ED, French SS. 2015. The role of corticosterone and toxicity in the antipredator behavior of the Rough-skinned Newt (*Taricha granulosa*). *General and Comparative Endocrinology*. 213:59-64.

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Dr. Gareth Hopkins
Research Fellow
School of BioSciences
The University of Melbourne

University of Virginia
Department of Biology

January 24, 2016

Ms. Lorin A. Neuman-Lee
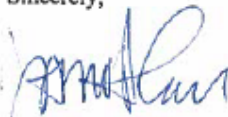Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Lorin,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Neuman-Lee LA, Brodie, Jr. ED, Hansen T*, Brodie III ED, and French SS. In press. To stress or not to stress: Physiological responses to tetrodotoxin in resistant gartersnakes vary by sex. *Comparative Biochemistry and Physiology: Part A.*

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Edmund D. Brodie III
B. F. D. Runk Professor, Biology
Director, Mountain Lake Biological Station

January, 25 2016

Ms. Lorin A. Neuman-Lee
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Lorin,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

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In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Tyler Hansen
Undergraduate Student
Utah State University

March 16, 2016

Ms. Lorin A. Neuman-Lee
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Lorin,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Neuman-Lee LA, Brodie, Jr. ED, Hansen T*, Brodie III ED, and French SS. In press. Comparing the natural and anthropogenic sodium channel blockers tetrodotoxin and indoxacarb in gartersnakes. *Journal of Experimental Zoology: Part A*

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Edmund Brodie III
Professor
Department of Biology
University of Virginia

January, 26 2016

Ms. Lorin A. Neuman-Lee
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Lorin,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Neuman-Lee LA, Carr J, Vaughn K*, French SS. 2015. Physiological effects of polybrominated diphenyl ether (PBDE-47) on pregnant gartersnakes and resulting offspring. *General and Comparative Endocrinology.* 219: 143-151.

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

James Carr
Professor
Department of Biological Sciences
Texas Tech University

April 21
March, _____ 2016

Ms. Lorin A. Neuman-Lee
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Lorin,

As the supervisor for Katelyn Vaugh, I certify that she was not the primary contributor to this work. In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation for the following paper:

Neuman-Lee LA, Carr J, Vaughn K*, French SS. 2015. Physiological effects of polybrominated diphenyl ether (PBDE-47) on pregnant gartersnakes and resulting offspring. *General and Comparative Endocrinology*. 219: 143-151.

Sincerely,

Dr. James Carr
Professor
Department of Biological Sciences
Texas Tech University

February 1, 2016

Ms. Lorin A. Neuman-Lee
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Lorin,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Neuman-Lee LA, Fokidis B, Smith GD, Spence A*, Van der Walt M*, French SS. 2015. Interactions between food restriction and chronic stress influence investment in immune function in gartersnakes. *Functional Ecology*. 29: 1453-1462.

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Geoff Smith
PhD Candidate
Department of Biology
Utah State University

January, 24 2016

Ms. Lorin A. Neuman-Lee
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Lorin,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Neuman-Lee LA, Fokidis B, Smith GD, Spence A*, Van der Walt M*, French SS. 2015. Interactions between food restriction and chronic stress influence investment in immune function in gartersnakes. *Functional Ecology.* 29: 1453-1462.

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Marilize Van der Walt
DVM Candidate
School of Veterinary Medicine
Utah State University

January, 30 2016

Ms. Lorin A. Neuman-Lee
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Lorin,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Neuman-Lee LA, Fokidis B, Smith GD, Spence A*, Van der Walt M*, French SS. 2015. Interactions between food restriction and chronic stress influence investment in immune function in gartersnakes. *Functional Ecology*. 29: 1453-1462.

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Austin Spence
PhD Student
Department of Ecology and Evolution
University of Connecticut

January 27, 2016

Ms. Lorin A. Neuman-Lee
Department of Biology
Utah State University
Logan, Utah, USA
84322-5305

Dear Lorin,

As a coauthor on the following manuscript, I grant you my permission to use this work in your dissertation:

Neuman-Lee LA, Fokidis B, Spence A*, Van der Walt M*, Smith GD, Durham S, French SS. 2015. Interactions between food restriction and chronic stress influence investment in immune function in gartersnakes. *Functional Ecology*. 29: 1453-1462.

In my opinion, you were the primary contributor to this work, which constituted independent and creative effort in design, implementation, and manuscript preparation.

Sincerely,

Susan Durham
Programmer/Analyst
Utah State University Ecology Center

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Lorin A. Neuman-Lee

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Utah State University

Logan, UT 84322

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lorin215@gmail.com

Education

PhD   Spring 2016

Biology, Utah State University
Advisor: Susannah French
Dissertation: *Endocrine, immune, and reproductive responses to natural and anthropogenic stressors in gartersnakes*

Master's of Science   Fall 2010

Biological Sciences, Eastern Illinois University
Advisors: Stephen Mullin and Karen Gaines
Thesis: *Multiple endpoints of endocrine disruption in gravid watersnakes (Colubridae: Nerodia) as a function of ingestion of a common herbicide*

Bachelor's of Science   Spring 2008

English, Iowa State University
Emphasis in Rhetorical Analysis

Bachelor's of Science   Fall 2007

Biology, Iowa State University
Advisor: Fredric Janzen

Publications

*Denotes undergraduate

Refereed Journal Articles

Skinner HM*, Durso AM, **Neuman-Lee LA**, Mueller SD*, and French SS. Effect of energy manipulation and diet complexity on life history strategies in Side-blotched lizards (*Uta stansburiana*). In review.

Skinner HM*, Smith GD, **Neuman-Lee LA**, and French SS. Energy allocations vary across years and an animal's reproductive life. In review.

Ranglack D[†], **Neuman-Lee LA**[†], French SS, and du Toit J. Using fecal glucocorticoids to explain unusual habitat selection patterns in free-ranging bison with high conservation value. In review.
[†]Authors contributed equally to this study

Spence AR*, Hopkins GR, **Neuman-Lee LA**, Smith GD, Brodie, Jr. ED, and French SS. Detrimental effects of zinc oxide nanoparticles on amphibian life stages. In review.

Hopkins GR., Brodie, Jr. ED, Mohammadi S, Brusch IV GA, **Neuman-Lee LA**, Hopkins ZM*, and French SS. In Press. Tidal newts: Physiological responses to salinity vary with proximity to the ocean in a coastal amphibian. *Physiological and Biochemical Zoology*.

Neuman-Lee LA, Brodie, Jr. ED, Hansen T*, Brodie III ED, and French SS. In press. To stress or not to stress: Physiological responses to tetrodotoxin in resistant gartersnakes vary by sex. *Comparative Biochemistry and Physiology: Part A.*

Neuman-Lee LA, Brodie, Jr. ED, Hansen T*, Brodie III ED, and French SS. 2016. Comparing the natural and anthropogenic sodium channel blockers tetrodotoxin and indoxacarb in gartersnakes. *Journal of Experimental Zoology: Part A.*

Mohammadi S, Brodie, Jr. ED, **Neuman-Lee LA**, Savitzky AH. 2016. Mutations to the cardiotonic steroid binding site of Na+/K+-ATPase confer high level of resistance to gamabufotalin in a natracine snake. *Toxicon*. 114: 13-15.

Neuman-Lee LA, Fokidis B, Spence A*, Van der Walt M*, Smith GD, Durham S, French SS. 2015. Interactions between food restriction and chronic stress influence investment in immune function in gartersnakes. *Functional Ecology*. 29: 1453-1462.

Neuman-Lee LA, Carr J, Vaughn K*, French SS. 2015. Physiological effects of polybrominated diphenyl ether (PBDE-47) on pregnant gartersnakes and resulting offspring. *General and Comparative Endocrinology*. 219: 143-151.

Neuman-Lee LA, Stokes AN, Greenfield S*, Hopkins G, Brodie ED, Jr., French SS. 2015. Effects of antipredator behavior and tetrodotoxin concentrations on stress response in Rough-skinned newts. *General and Comparative Biology*. 213:59-64.

Boone MD, Bishop CA, Boswell LA, Brodman RD, Burger J, Davidson C, Gochfeld M, Hoverman JT, **Neuman-Lee LA**, Relyea RA, Rohr JR, Salice C, Semlitsch RD, Sparling D, and Weir S. 2014. Pesticide regulation amid the influence of industry. *Bioscience*. doi: 10.1093/biosci/biu138

Neuman-Lee LA and French SS. 2014. Wound healing reduces stress-induced immune changes: Evidence for immune trade-offs in the side-blotched lizard. *Journal of Comparative Physiology B*, 1-7

Neuman-Lee LA, Gaines KF, Baumgartner KA*, Voorhees JM*, Mullin SJ. 2014. Assessing holistic effects of atrazine ingestion on gravid Northern Watersnakes (*Nerodia sipedon*) and their offspring. *Environmental Toxicology*. 29(9): 1072-1082 doi: 10.1002/tox.21837.

Warner DA, Jergenson A*, **Neuman-Lee LA**, Janzen FJ. 2014. Swimming against the tide: resilience of a riverine turtle to extreme environmental events. *Biology Letters* 10(3) doi: 10.1098/rsbl.2013.0782

Stokes AN, Ducey PK, **Neuman-Lee LA**, Hannifin CT, French SS, Pfrender ME, Brodie ED III, Brodie ED, Jr. 2014. Confirmation and distribution of tetrodotoxin for the first time in terrestrial invertebrates: Two terrestrial flatworm species (*Bipalium adventitium* and *Bipalium kewense*). *PLoS ONE*. 9(6) doi: 10.1371/journal.pone.0100718.

Neuman-Lee LA, Hopkins GR, Brodie ED, Jr., French SS. 2013. Sublethal contaminant exposure alters behavior in a common insect: Important implications for tropic transfer. *Journal of Environmental Science and Health, Part B*. 48(6): 442-448.

Neuman-Lee LA, Durso AM, Kiriazis NM*, and Mullin SJ. 2013. Differential habitat use by Common Watersnakes (*Nerodia sipedon*). *IRCF Reptiles & Amphibians* 20(4): 166-171.

French SS and **Neuman-Lee LA**. 2012. Novel ex vivo method for microbiocidal activity across vertebrate species. *Biology Open* 1: 482-487.

Neuman-Lee LA and Janzen FJ. 2011. Atrazine exposure impacts behavior and survivorship of neonatal turtles. *Herpetologica* 67(1): 23-31.

Neuman-Lee LA, McGaugh SE, Pfrender M, and Janzen FJ. 2011. Using mitochondrial DNA to determine the identity and origin of a gartersnake found in Alaska. *Journal of Herpetology* 45(1):63-65.

In preparation

Neuman-Lee LA, Skinner H*, and French SS. Synergistic effects of polybrominated diphenyl ether-47 and chronic stress on gartersnakes.

Neuman-Lee LA and Janzen FJ. Comparison of Softshell (*Apalone*) and Map Turtle (*Graptemys*) Embryonic Development during early atrazine exposure.

Neuman-Lee LA, Strickland JT, Thol S, Janzen FJ. TREE: Building an Effective Recruitment Program for Ecology.

Ryan M*, **Neuman-Lee LA**, and French SS. Temperature preferences and behavioral fever in African House Snakes.

Smith GD, **Neuman-Lee LA**, Greenfield S*, and French SS. Temperature effects on wound healing profiles in the Rough-skinned newt.

Stevenson ET, Gese EM, French SS, and **Neuman-Lee LA**. Degradation of glucocorticoid metabolites in coyote scats: implications for field studies.

Van der Walt M*, Smith GD, **Neuman-Lee LA**, and French SS. Effect of noise disturbance on lizard physiology.

Van der Walt M*, **Neuman-Lee LA**, Smith GD, and French SS. Conspecific housing in lizards: costs and benefits.

Webb A, Neuman-Lee LA, Lin SM, and French SS. Immune and endocrine function in *Takydromus* lizards of Taiwan.

Geographic Distribution Notes
Neuman-Lee LA and Durso AM. 2011. *Graptemys pseudogeographica* (False Map Turtle) Carroll & Whiteside Counties, Illinois. Geographic Distribution. Herpetological Review. 42(1):110.

Neuman-Lee LA and Durso AM. 2011. *Graptemys geographica* (Northern Map Turtle) Whiteside County, Illinois. Geographic Distribution. Herpetological Review. 42(1):110.

Durso AM and **Neuman-Lee LA**. 2011. *Chelydra serpentina* (Snapping Turtle) Whiteside County, Illinois. Geographic Distribution. Herpetological Review. 42(1):110.

Other Publications
Neuman-Lee L. 2008. A Day at Turtle Camp. *The Big Muddy: A Journal of the Mississippi River Valley* 8(1):40-47.

Awards and Honors

2016   College of Science Researcher of the Year, Utah State University
2016   Biology Graduate Researcher of the Year, Utah State University
2013   Biology Graduate Teacher of the Year, Utah State University
2012   James A. and Patty MacMahon Ecology Graduate Student Research
          Award, Utah State University
2010   Distinguished Graduate Student Award, Eastern Illinois University
2010   Graduate Showcase Recipient, Eastern Illinois University
2009   Graduate Research Award, 2nd Runner-up, Herpetologists' League
2009   Stephen Jay Gould Award (*Darwin's Rhetorical Model: Its effectiveness
          and continued use*), Eastern Illinois University
2008   Cardinal Key Honor Society (<1% of ISU Senior Class), Iowa State
University

Grants and Fellowships

2013   Doctoral Dissertation Improvement Grant, Utah State University
2010-2011 Vice President Research Fellowship, Utah State University
2010   Graduate Student Investigator Award, Eastern Illinois University
2010   Williams Travel Award, Eastern Illinois University
2009   Graduate Student Research Award, The Wildlife Society's Toxicology
          Working Group
2009   Presidential Graduate Assistantship, Eastern Illinois University
2009   Williams Travel Award, Eastern Illinois University
2005   Renewing Earth and Its People (REAP)

Invited Talks

2012   "Ecological and physiological effects of the herbicide atrazine." Franklin
          and Marshall College; Lancaster, PA
2011   "Holistic effects of atrazine on pregnant watersnakes and their offspring."
          Biology Department Seminar; University of Utah; Salt Lake City, UT
2008   "Planting a TREE Program: A Practical Model." Ecological Society of
          America Annual Meeting; Milwaukee, WI (with J Strickland, J Maciel, S
          Thol, and F Janzen)

Conference Activity/Participation
*Denotes undergraduate

Oral Presentations

2016   Smith GD, **Neuman-Lee LA**, Webb AC, Denardo DF, Angilletta MJ, and French SS. "Lizards downregulate their metabolism in response to an immune challenge: implications for energy status." Society of Integrative and Comparative Biology. Portland, OR.

2015   **Neuman-Lee LA**, Brodie, Jr. ED, and French SS. "Physiological consequences of evolution: Quantifying the costs of adaptive resistance to tetrodotoxin in gartersnakes. Society of Integrative and Comparative Biology. West Palm Beach, FL.

2015   Smith GD, Durso AM, **Neuman-Lee LA**, and French SS. "The town lizard and the country lizard: the physiological ecology of urbanization in*Uta stansburiana."* Society of Integrative and Comparative Biology. West Palm Beach, FL.

2015   Spence AR*, Hopkins GR, **Neuman-Lee LA**, Brodie, Jr. ED, French SS. "Effects of chronic and acute exposure to ZnO nanoparticles across life-history stages in a caudate amphibian (*Taricha granulosa*). Society for Integrative and Comparative Biology; West Palm Beach, FL.

2014   Mohammadi S, Savitzky AH, **Neuman-Lee L**, Petschenka G. "Molecular and Physiological Mechanisms of Bufadienolide-resistance in Toad-Eating Snakes." Joint Meeting of Ichthyologists and Herpetologists. Chattanooga, TN.

2014   **Neuman-Lee LA**, Smith GD, and French SS. "Interactive effects of food restriction and restraint stress on stress reactivity, immune function, and energy metabolite use and storage in snakes." Society of Integrative and Comparative Biology. Austin, TX.

2014   Durso AM, Smith GD, **Neuman-Lee LA,** and French SS. "Using labeled nutrient tracers to reveal resource allocation in lizards with competing needs*."* Society for Integrative and Comparative Biology. Austin, TX.

2014   Smith GD, **Neuman-Lee LA**, Durso AM, and French SS. 2014. "Investment between reproductive and immune systems varies with latitude and time in *Uta stansburiana*." Society for Integrative and Comparative Biology. Austin, TX.

2011   **Neuman-Lee LA**, Gaines KF, Baumgartner KA*, Voorhees J*, and Mullin SJ. "Using multiple endpoints to determine the effects of atrazine ingestion on gravid female watersnakes and their offspring." Society for Integrative and Comparative Biology; Salt Lake City, UT.

2010   **Neuman-Lee LA**, Gaines KF, and Mullin SJ. "Estradiol levels in watersnakes (Colubridae: Nerodia) during gestation as a function of exposure to ingested atrazine." Joint Meeting of Ichthyologists and Herpetologists; Providence, RI.

2010   **Neuman-Lee LA**, Mullin SJ and Gaines KF. "Atrazine during pregnancy: Its impacts on viviparous reptile mothers and young." Midwest Ecology and Evolution Meetings; Ames, IA.

2009   **Neuman-Lee LA** and Janzen F. "Examining the effects of atrazine on embryos of turtles from two deeply divergent Cryptodiran families (Emydidae and Trionychidae)." Joint Meeting of Ichthyologists and Herpetologists; Portland, OR.

2008   **Neuman-Lee L.** "Short term exposure to herbicide yields long term effects in Map Turtles (*Graptemys*)." Ecological Society of America Annual Meeting; Milwaukee, WI.

2008   **Neuman-Lee LA**. "Short term exposure to herbicide yields long term effects in Map Turtles (*Graptemys*)." Undergraduate Research Symposium; Iowa State University.

Poster Presentations

2016   **Neuman-Lee LA** and French SS. *Ecoimmunology tools in gartersnakes*. Society of Integrative and Comparative Biology. Portland, OR.

2015   Van der Walt M*, Smith GD, **Neuman-Lee LA**, and French SS. *Group housing and stress in Side-Blotched Lizards.* Society for Integrative and Comparative Biology; West Palm Beach, FL.

2015   French SS, **Neuman-Lee LA**, Greenfield SM*. Effects of corticosterone and ACTH on the unken reflex in Rough-skinned newts, *Taricha granulosa*. Society for Integrative and Comparative Biology; West Palm Beach, FL.

2015   Van der Walt M*, Smith GD, **Neuman-Lee LA**, and French SS. Lonely lizards: individual housing causes physiological change in side-blotch

lizards (*Uta stansburiana*). Association of Zoos and Aquariums; Salt Lake City, UT.

2014   Durso AM, Smith GD, **Neuman-Lee LA,** and French SS. *Using labeled nutrient tracers to reveal resource allocation in lizards with competing needs.* Joint Meeting of Ichthyologists & Herpetologists. Chattanooga, TN.

2014   Hopkins GR., Brodie, Jr. ED, **Neuman-Lee LA**, Stopher ZM*, and French SS. *Tidal newts: Evolution in a stressful environment.* Genomes to Biomes—1st Joint Conference of the Canadian Society for Ecology and Evolution, Canadian Society of Zoologists, and the Society of Canadian Limnologists, Montreal, Quebec, Canada.

2013   Hopkins GR., Brodie, Jr. ED, **Neuman-Lee LA**, Stopher ZM*, and French SS. *Tidal newts: Evidence for local adaptation to an osmotically stressful environment.* Annual Meetings of the Society for the Study of Evolution & American Society of Naturalists, Snowbird, Utah.

2012   **Neuman-Lee LA** and French SS. *Effects of prenatal exposure to a flame retardant on survival and growth in juvenile Western Terrestrial Gartersnakes (*Thamnophis elegans*).* World Congress of Herpetology, Vancouver, British Columbia, Canada.

2011   **Neuman-Lee LA** and French SS. 2011. *Endocrine, immune, and behavioral responses to flame retardant exposure in gartersnakes.* 7th International Symposium on Amphibian and Reptilian Endocrinology and Neurobiology. Ann Arbor, MI.

2011   Gross IP*, **Neuman-Lee LA**, Durso AM, and Mullin SJ. *Assessing the relationship between parasite loads & limb deformities in Small-mouthed Salamanders (Caudata: Ambystomatidae).* Joint Meeting of Ichthyologists & Herpetologists. Minneapolis, MN.

2010   Voorhees JR*, **Neuman-Lee LA**, Mullin SJ, and Gaines KF. 2010. *Lipid concentrations in neonate watersnakes (Colubridae: Nerodia) as a function of in utero exposure to atrazine.* Joint Meeting of Ichthyologists and Herpetologists, Providence, RI.

2010   Baumgartner KA*, Mullin SJ and **Neuman-Lee LA**. *The Effect of atrazine on scale-row symmetry during the development of neonate watersnakes (Colubridae:* Nerodia*).* Midwest Ecology and Evolution Meetings, Ames, IA.

2009   Neuman-Lee LA, Strickland J, Thol S, Janzen F. *Planting a TREE: Exposing high school students to ecological research with reptiles*. Joint Meeting of Ichthyologists and Herpetologists. Portland, OR.

2009   Kiriazis NM*, **Neuman-Lee LA** and Mullin SJ. *Population structure and morphometrics of Northern Watersnakes (*Nerodia sipedon*) in an anthropogenic habitat*. Joint Meeting of Ichthyologists and Herpetologists. Portland, OR.

2009   **Neuman-Lee LA** and Janzen F. *Using DNA sequencing to determine the identity and geographic origin of an unknown snake in Alaska*. Snake Ecology Group Meeting. Donnelly, ID.

2008   **Neuman-Lee LA**, Thol S, Strickland J, Janzen F. *The TREE Program: Bringing Ecological Research to Local Students*. Partners in Amphibian and Reptile Conservation (PARC). Bluegrass, IA.

2008   **Neuman-Lee LA** and Janzen F. *Feeding behavior altered by short-term atrazine exposure during incubation in map turtles (Graptemys)*. Research at the Capitol. Des Moines, IA.

2007   **Neuman-Lee LA** and Janzen F. *Survivability and Recruitment in Illinois Population of Male Painted Turtles (C. picta)*. Science with Practice. Ames, IA.

2007   **Neuman-Lee LA**, Thol S, Strickland J, Janzen F. *The TREE Program: Bringing Ecological Research to Local Students*. Research Experience for Undergraduates (REU). Ames, IA.

2006   **Neuman-Lee LA** and Janzen F. *Effects of Atrazine on Map Turtle (Graptemys) Development and Behavior*. Society of Integrative and Comparative Biology. Orlando, FL.

2004   **Neuman-Lee LA** and Janzen F. *The Effects of Atrazine on the Performance, Survival, and Behavior of Embryonic Map Turtles (Graptemys).* Society of Integrative and Comparative Biology, New Orleans, LA.

Teaching Experience

Utah State University
2015 (Fall)   Comparative Physiology Lecture, Instructor

2015 (Spring)	Principles of Genetics, Guest lecturer on Mendelian genetics, sex determination and sex-linked characteristics (3 lectures)
2014 (Spring)	Human Physiology Lab (4 sections), TA
2013 (Fall)	Comparative Animal Physiology Lab (2 sections), Instructor—designed and developed 3 new labs on crayfish metabolism, snake stress and immune physiology, and osmoregulation in newts and invertebrates
2013 (Spring)	Introductory Lab for Biology Majors II (3 sections), TA
2012 (Fall)	Comparative Animal Physiology Lab (2 sections), Instructor
2012 (Spring)	Principles of Genetics, Guest lecturer on developmental, evolutionary, immuno-, and population genetics (4 lectures)
2011 (Fall)	Introductory Lab for Biology Majors I (3 sections), TA

Eastern Illinois University

2010 (Spring)	Biology for Non-Majors Lab (2 sections), Instructor
2009 (Fall)	Biology for Non-Majors Lab (2 sections), Instructor
2009 (Spring)	Herpetology Lab (1 section), TA
2009 (Spring)	Biology for Majors Lab (1 section), TA
2008 (Fall)	Ecology Lab (2 sections), TA

Mentoring Experience

2007-2011	Coordinator and designer of Turtle Camp Research and Education in Ecology (TREE) Program, Iowa State University—mentored 20 high school students and 6 undergraduate students through this project

Mentored Students
2010- present (Utah State University)

Holden Brown—Organ weights in chemically-treated snakes
Landon Felhberg (Teaching scholar)—Glucose levels in recently emerged snakes
Sydney Greenfield—Temperature effects of wound healing in newts
Tyler Hansen—TTX and snake resistance
Heather Skinner (veterinarian student)—Maternal investment and immunity in lizards
Georgia Kosmala (Brazilian visiting scholar)—Corticosterone levels in larval newts
Michael Ryan—Effects of temperature on immune function in house snakes
Eric Sims—Organ weights in chemically-treated snakes
Austin Spence—Exploring the effect of construction on snake populations
Marilize Van der Walt—Determining how noise and conspecifics affect lizard stress
Eleanor Watson—Conspecific choice of scent in male and female snakes

2008-2010 (Eastern Illinois University)
>   Kyle Baumgartner—Fluctuating asymmetry in watersnake neonates
>   Iwo Gross—Animal care and laboratory procedures
>   Nicholas Kiriazis—Differential habitat selection in watersnakes
>   Mary Jo Kocher—Animal care and laboratory procedures
>   Jamie Voorhees—Lipid content in livers of atrazine-treated watersnakes

2008 (Iowa State University)
>   Kyndra Lundquist—Hatching synchrony in two species of freshwater turtle

Research Experience

2010-present
Dissertation Research Understanding the stress response in reptiles and its effect on endocrine and immune function using hormone and innate immune assays (w/ Susannah French, Utah State University).

2008-2010
Master's Research Examining link between pregnant female Northern Watersnakes (*Nerodia sipedon*) treated with an endocrine disruptor (atrazine), estradiol fluctuations, and neonate development (w/ Stephen Mullin and Karen Gaines, Eastern Illinois University).

2002-2008
Undergraduate Research Assistant Studied the impact of the common herbicide, atrazine, on the growth, behavior, and development of the turtles. Worked at "Turtle Camp" in the summers of 2003, 2004, 2006, 2007 and 2008 processing, measuring, marking, trapping, and collecting blood samples from the turtles *Chrysemys picta, Emys blandingii, Terrapene ornata, Chelydra serpentina,* and *Apalone mutica.* Monitored the nesting habits of *C. picta* and processed the laid nests and subsequent eggs. Extracted mDNA and sequenced two different sites (cytb and ND2) of a species of snake (*Thamnophis*) to determine its origin and phylogeographic relationship (w/ Fredric Janzen, Iowa State University).

2007
Science with Practice Research Assistant Examined population dynamics of painted turtle males using Program MARK (w/ Fredric Janzen, Iowa State University)

2007
NSF Research Experience for Undergraduates Research Assistant Trapped and marked turtle and snake individuals for long-term capture-mark-recapture projects (w/ Fredric Janzen, Iowa State University)

2005	*Fundación SELVA* and *New Community Project Intern* Catalogued medicinal plants with the aid of two indigenous shamans in Ecuadorian rainforest
2004	*Program for Women in Science and Engineering Research Assistant* Performed feeding trials on atrazine-treated turtles *Graptemys ouachitensis* and *Graptemys pseudogeographica* (w/ Fredric Janzen, Iowa State University)
2002-2004	*Undergraduate Mentoring in Environmental Biology Research Assistant*

Service to Profession

2009-2015	Chair, Herpetologists' League Graduate Committee
2010	Committee Member, Meritorious Teaching Award in Herpetology, given by 3 national herpetology societies
2011	Committee Member, Meritorious Teaching Award in Herpetology, given by 3 national herpetology societies
2006-2008	Student Representative and founder, Iowa State University Chapter of Strategies for Ecology Education, Development and Sustainability (SEEDS) Program (Ecological Society of America)
2005-2007	Chair and co-founder, Environmental Committee, Iowa State University
2003-2006	Mentor, Biology Education Success Team, Iowa State University

Reviewer

African Journal of Biotechnology; Biology Open; Bulletin of Environmental Contamination and Toxicology; Canadian Journal of Zoology; Ecotoxicology; Ethology, Ecology, and Evolution; Functional Ecology; Herpetologica; Journal of Experimental Zoology, Journal of Herpetology; Mammalian Biology; PLoS ONE

Service to University

2014	Biology Graduate Student Association Outreach and Social Committee, Utah State University
2012	Faculty Search Committee, Utah State University
2006	Senator (elected), Government of the Student Body, Iowa State University
2006-2008	Vice-President (elected), Sigma Tau Delta English Honor Society, Iowa State University

Community Outreach and Education

2016	Stokes Nature Center (outreach program)
2015	Venture High School (research adviser)
2015	Thomas Edison Science Fair Judge
2015	Stokes Nature Center (outreach program)
2014	Box Elder High School (guest speaker)
2014	Stokes Nature Center (outreach program)
2014	Science Unwrapped (outreach program)
2014	Cache Youth Resource Center (outreach program)
2014	Thomas Edison Science Fair Judge
2014	Native American Pow Wow at USU (outreach program)
2013	Science Unwrapped (outreach program)
2013	No Child Left Indoors (outreach program)
2013	Bear River Migratory Refuge (outreach program)
2012	No Child Left Indoors (outreach program)
2012	Bear River Migratory Refuge (outreach program)
2011	Edith Bowen Elementary School (outreach program)
2010	Celebration! at Eastern Illinois University (outreach program)
2009	Celebration! at Eastern Illinois University (outreach program)
2009	Thomson Public Library (outreach program)
2008	Thomson Public Library (outreach program)
2007	Boy Scouts of America (outreach program)
2007	Thomson Public Library (outreach program)
2006	Wildlife Care Clinic (volunteer—1 year)
2006	Boy Scouts of America (outreach program)
2006	Thomson Public Library (outreach program)

Research in the Media

2014   USU scientist discusses concerns with EPA's regulation of pesticides. The Herald Journal. September 11, 2014 http://news.hjnews.com/allaccess/usu-scientist-discusses-concerns-with-epa-s-regulation-of-pesticides/article_38d5de4e-3941-11e4-85a7-cbe3b9a9d55d.html

2014   Utah Public Radio. September 5, 2014 (Interviewer Evan Hall)

2014   Does the EPA favor industry when assessing chemical dangers? Newsweek. September 3, 2014. By Zoe Schlanger. http://www.newsweek.com/does-epa-favor-industry-when-assessing-chemical-dangers-268168

2010   In field or backyard, frogs face threats. ScienceNews 178(6): 28 by S. Milius. online at http://www.sciencenews.org/view/feature/id/62649/title/In_field_or_backyard,_frogs_face_threats

<u>Honor Society Memberships</u>

Sigma Xi Scientific Research Society
Phi Sigma Biological Sciences Honor Society
Sigma Tau Delta English Honor Society

<u>Society Memberships</u>

The Society for Integrative and Comparative Biology, Society of Environmental Toxicology and Chemistry, Herpetologists' League, American Institute of Biological Sciences, Sigma Xi