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PHYSIOLOGICAL CONSEQUENCES OF COMPENSATORY GROWTH: A LOOK AT SNAKE SPECIES EXHIBITING DIFFERENTIAL SEXUAL SIZE DIMORPHISM

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
Department of Biology

Neil B. Ford, Ph.D., Committee Chair

College of Arts and Sciences

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Although many life-history traits are inflexible, extrinsic factors are likely responsible for variation in attributes such as growth rates and ultimately adult body size. Nutritional stress during natal periods, in particular, has significant long-term consequences for adult characteristics. It is thought that poor natal nutrition leads to either the diversion of subsequent energy intake into compensatory growth or delayed maturation. Diversion of energy, to achieve compensation, however does not come without consequence; immune defense is a resource-demanding activity, which can trade-off with traits such as growth. Relatively little is known about the intra- or interspecific variation in immunological capabilities, especially in the context of the effects of stress on immune function. Stress induced levels of glucocorticoids (corticosterone) cause a shift in physiological parameters such that self-maintenance and survival processes are prioritized; by redirecting resources, corticosterone and thus stress especially at chronic levels is generally considered immunosuppressive. I looked at the effects of poor natal

nutrition and the potential for compensation on types of immune defense and corticosterone levels in checkered garter and corn snakes, species with different sexual dimorphisms. The effects of accelerated growth rates, following a period of suppressed growth, on immunological function and stress were negligible. This suggests that either snakes are robust animals that can maintain health at low levels of food intake, or that immune function parameters are not downgraded when excess energy is allocated towards compensation (partial or complete). Immune function is thus suggested to be evolutionarily adapted to be maintained during periods of stress.

CHAPTER ONE

INTRODUCTION

An animal's size influences many important aspects of its interaction with its environment. The adult phenotype of an organism results from both its genetic makeup and from the environment's effect on the expression of those genes (Metcalfe and Monaghan, 2001). Theoretically, an individual should match its phenotype to the current environmental conditions to maximize fitness (Roff, 2002). Though many life-history traits are inflexible, extrinsic factors such as temperature, predation risk or low resource availability are likely responsible for variation in attributes such as growth rates and ultimately adult body size (Radder et al., 2007). Nutritional stress during natal periods, in particular, has significant long-term consequences for adult characteristics (Kubicka and Kratochvil, 2009).

Nutritional stress can be a result of insufficient time to acquire resources for those born late in a breeding season, being born into poor environmental conditions or due to poor parental provisioning in the egg or juvenile stages (Metcalfe and Monaghan, 2001). Food availability can also be unpredictable and highly variable in natural environments thus growth rates of individuals often respond accordingly (Radder et al., 2003). Even when subsequent improvement in prey availability allows for compensation for prior reduced growth, an early-restricted diet can profoundly affect adult phenotypes and fitness.

Following a period of nutritional stress, when resources become plentiful again,

individuals may respond in several ways. Individuals may not respond at all and continue to grow on a "normal" trajectory from a smaller size at age, may exhibit faster-than-normal growth immediately following the restricted period, or may adopt a growth strategy that involves increasing growth rate at a later point (Mangel and Munch, 2005). Thus, responses to poor natal nutrition are either the diversion of subsequent energy intake into compensatory growth or delayed maturation (Ford and Seigel, 1989a,b; Metcalfe and Monaghan, 2001; Byars, et al., 2010; Ujvari, et al., 2011). It is expected that selection will favor growth compensation unless compensation is, in itself, too costly (Metcalfe and Monaghan, 2001). Growth rates are flexible and are usually regulated at optimal rather than maximal rates, such that rates can be increased when selective pressures favor an increase in overall size (Arendt, 1997; Metcalfe and Monaghan, 2001; Mangel and Munch, 2005; Stoks et al., 2006). The diversion of energy to achieve compensatory growth after a period of stress however does not come without morphological, behavioral, and/or physiological consequences.

Many studies have examined the consequences of compensatory growth, and a number of outcomes have been identified. A study on brown tree frogs (*Litoria ewingii*) found decreased pre- and postmetamorphic survival rates along with premetamorphic swimming speed deficiencies and an extended larval period in those individuals nutritionally stressed in early life stages (Hector et al., 2012). Other documented morphological trade-offs include the preservation of body condition at the cost of skeleton size in teleost fishes (Ali et al., 2003), and the maintenance of abdomen size at the cost of thorax size in the caddis fly (*Glyphotaelius pellucidus*) (Stevens et al., 2000). Dietary stress received specifically during phases of development can also proportionally

affect the allocation of resources to specific components of mass such as fat, protein content and relative wing size in insects instead of maintaining a smaller overall size (Ojeda-Avila et al., 2003; Boggs and Freeman, 2005; Stoks et al., 2005). Nutritional stress and energy diversion to compensatory growth has also been shown to cause a reduction in HVC volume in the song sparrow (*Melospiza melodia*) (Macdonald et al., 2004), decreased and shorter song bouts in the European Starling (*Sturnus vulgaris*) (Buchanan et al., 2003) and an overall reduction in cognitive function in zebra finches (*Taeniopygia guttata*) (Fisher et al., 2006). On a behavioral level, compensatory growth from nutritional stress has been proven to increase foraging behavior in butterflies (*Pararge aegeria*) despite increased abundance of predators (Gottard, 2000) and impair both escape capabilities and routine locomotor speed in fish (*Gasterosteus aculeatus*) (Álvarez and Metcalfe, 2005; 2007). Physiologically it's been shown that compensatory growth is achieved after periods of stress by increased metabolic rates at the cost of decreased energy storage potential in the damselfly (*Lestes viridis*) (Stoks et al., 2006). Other physiological trade-offs documented include decreases in litter sizes, though not litter production rates or investment in offspring size, in Trinidadian guppies (*Poecilia reticulate*) (Auer et al., 2010), and overall decreases in lifespan at the cost of growth to maintain reproductive investment in the three-spined stickleback (*Gasterosteus aculeatus*) (Inness and Metcalfe, 2008). Immune defense is also an example of a resource-demanding activity that can trade-off with growth and reproduction (Norris and Evans, 2000). Studies by Buchanan et al. (2003) found suppressed humoral responses in the European starling following catch-up growth.

COMPENSATORY GROWTH

The term "compensatory growth" or "catch-up growth" describes both the pattern and the process by which an organism grows more quickly after a period of reduced growth than would be expected in the absence of restriction. In other words, compensatory growth is a phase of accelerated growth that occurs when normal nutrition is available after a period of growth inhibition (Ali et al, 2003; Metcalf and Monaghan, 2001). The accelerated growth is typically at a higher rate than expected in the absence of growth inhibition (Nicieza and Alvarez, 2009).

The underlying physiological mechanisms, which divert resources to either compensatory growth or into delaying maturation, has been poorly explained in studies that demonstrate the effect of early nutritional stress on adult traits. Compensatory growth can be accomplished with a hyperphagic response when resource levels return (Ali et al., 2003) or by a change in resource allocation to growth at the expense of other activities such as immune function (Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; Soler et al., 2003). Hyperphagia is a rate of food consumption significantly higher than that shown by individuals that have been continuously offered an *ad libitum* diet (Russell and Wooton, 1993; Ali et al. 2003).

During phases of compensatory growth, the mass gained by an organism tends to be biased toward a more rapid accumulation of fat rather than non-adipose tissue (Metcalfe and Monaghan, 2003). Increased adiposity can persist beyond the phase of compensatory response (Dulloo et al., 2002). This response could be an evolutionary adaptation to a perceived risk of starvation, where an animal responds to a food shortage by storing more fat, and any negative effects of carrying excess fat may be outweighed by

the reduced risks of dying of food shortage (Cuthill et al., 2000). Alternatively, a change in body composition due to increased adiposity may simply be a physiological constraint on rapid protein growth where accumulation of protein mass lags behind that of fats (Metcalfe and Monaghan 2003). Given that fast growth is associated with a shortened life span (Metcalfe and Monaghan, 2001; Metcalfe and Monaghan, 2003; Inness and Metcalfe, 2008; Sparkman and Palacios, 2009) the question arises why an organism would invest energy into accelerating growth following food restriction. Two ecological advantages come from compensatory responses: short-term survival and reproductive success. Predation is often strongly linked to body size (smaller individuals being easier to predate) so rapid growth reduces the duration of the vulnerable period (Arendt, 1997). Also, the maximum amount of energy reserves that can be stored disproportionately increases with the size of the individual, thus larger individuals have a lower risk of dying of starvation during periods of food shortage (Ludsin and DeVries, 1997; Metcalfe and Monaghan 2003). In species where growth does not continue after reaching sexual maturity, a failure to compensate for early growth restriction can result in reduced fitness even if lifespans are long. This reduction in fitness is most often a result of being outcompeted for reproductive opportunities (Richner, 1992; Metcalfe and Monaghan 2003).

Poor nutritional conditions at natal stages can also affect one sex more severely than the other (Metcalfe and Monaghan, 2001; Le Galliard et al., 2005) such that typically the faster growing sex is more vulnerable to restricted food availability (Tschirren et al., 2003). These responses to natal nutritional stress are proposed to have different mechanisms, which are hypothesized to depend on whether selection favors

increased size at age of first reproduction or for early reproduction regardless of size. When increased size at age of first reproduction is selected for, smaller females that would have smaller or fewer offspring are selected against. The drive to compensate for small size may also differ between the sexes because many species exhibit sexual size dimorphism in adult size; for some species females grow larger than males and in others males reach a larger adult size. In males, larger size is advantageous in species that display intrasexual competition. In females size is an advantage for fecundity reasons, especially in species that exhibit indeterminate growth and indeterminate fecundity (Taylor and DeNardo, 2005).

Compensatory responses to growth restriction have been suggested to carry physiological (e.g. high metabolic rates that would more quickly deplete resources or allocate more resources into growth and less to energy storage), developmental (e.g. lower the degree of quality control with high probability for developing developmental errors), ecological (e.g. put an organism at a higher risk for predation) and immunological (e.g. decrease an individual's ability to defend against foreign agents or resist disease) costs (Gotthard, 2001). Mounting an immune response and maintaining a competent immune system is a resource-demanding process that can also be interchanged with growth, thermoregulation and reproduction when energy intake is reduced (Sheldon and Verhulst, 1996; Norris and Evans, 2000; Lochmiller and Deerenberg, 2000). During periods of rapid growth such as during compensatory growth, it has been predicted that innate immune constituents should prevail over induced acquired defenses. Innate immune constituents are less expensive to maintain than induced acquired defenses.

IMMUNITY

Animals face continuous threat from parasites and pathogens that can result in a considerable reduction in reproductive success and overall survival. In response to these threats, the organisms have evolved a complex series of behavioral, physical and physiological measures to attempt to minimize fitness costs stemmed from them. Among the physiological measures, the immune system is one of the major mechanisms in host survival (Lockmiller and Deerenberg, 2000). The immune system of vertebrates can be divided into two main divisions: non-specific innate immunity and specific acquired immunity. Innate immunity involves rapid development of responses to pathogens as a first line of defense; whereas acquired (or adaptive) immunity acts as a second line of defense and is more effective against repeated infection (Sparkman and Palacios, 2009). Components of both the innate and acquired divisions can further be classified as either constitutive (expressed at all times) or induced (expressed upon encounter of pathogens) (Schmid-Hempel and Ebert, 2003; Lee, 2006). Investment in constitutive innate and induced acquired immunity may vary according to the life-history characteristics and strategy of the organism in question; however, acquired immunity though more effective against repeated infections is also more costly to retain (Sheldon and Verhulst, 1996; Norris and Evans, 2000; Lee, 2006). Though the costs of constitutive innate immunity have not been definitively measured, the expense is thought to be comparatively low, because of the lack of a diversification process like that required for lymphocyte development, low rates of cell turnover when an immune response is not being mounted, and also the small tissue mass accounted for by constitutive innate cells and proteins (Klasing and Leshchinsky, 1999; Lee, 2006).

Early ecoimmunological interpretations of the trade-offs between resource-demanding traits, from which life-history theory is based, proposed that high investment in growth or reproduction would result in reduced investment in immune function; causing a trade-off with self-maintenance (Sheldon and Verhulst 1996; Norris and Evans 2000; Schmid-Hempel 2003). The traits of maintaining a functioning immune system and actually utilizing it are not easily quantified due to the integrated characteristics of the immune system with other physiological systems. Thus, actual attempts to exhibit the magnitude of cost of immunity have been limited. However, existing studies demonstrate that considerable nutritional costs are associated with the up-regulation of the immune system (Lochmiller and Deerenberg, 2000). For instance, a negative correlation between brood size and different components of immunity has been shown in three bird speicies: collared flycatchers (*Ficedula albicollis*), zebra finches (*Taeniopygia guttata*), and barn swallows (*Hirundo rustica*) (Nordling, 1998; Apanius et al., 1994, Saino, 1997). The consequences of this up-regulation of immunity to vertebrate survival suggest that selection should have favored a level of innate mechanisms of immunity over acquired mechanisms of immunity. Favoring innate immunity over acquired immunity suggests that a higher energetic cost is associated with acquired immunity and thus innate immunity would be preferentially selected for. Nutritional trade-offs between immunity and other nutrient-demanding processes have been the focus of more recent evolutionary investigations of immunology (Sheldon and Verhulst, 1996). Trade-offs between processes such as growth, thermoregulation or reproduction may be most pronounced within the acquired division of the immune system (Fearon and Locksley 1996) as shown by Stier et al. (2009) where they found that under stressful conditions in barn owl

nestlings (*Tyto alba*) inducible immune responses were negatively affected while constitutive innate immunity was maintained.

Recent interest in examining immunological parameters as a way to study a multitude of ecoimmunological hypotheses has resulted in the development of different techniques. Many of these techniques require relatively small blood samples, making them ideal for studies of natal periods where the study animal may be of small size. Constitutive innate immunity can be measured through natural antibodies, complement-mediated lysis and bactericidal competence of plasma, all of which are part of the first line of defense again invading microorganisms (Sparkman and Palacios, 2009). Natural antibodies are unique among immunoglobulin molecules because their presence does not require previous exposure to a particular antigen and react with various affinities to a wide variety of epitopes on macromolecular and particulate antigens including foreign red blood cells (Greenberg, 1985; Belperron, 2001; Matson et al., 2005). In other words, natural antibodies are non-specific defenses, recognizing a broad array of pathogens and promoting their opsonization and phagocytosis (Ochsenbein and Zinkernagel, 2000; Sparkman and Palacios, 2009). In addition, natural antibodies can activate the complement enzyme cascade, which results in the formation of killing complexes on the surface of invading pathogens, leading to their lysis (Ochsenbein and Zinkernagel, 2000; Sparkman and Palacios, 2009). Bactericidal competence is a measure of the intrinsic bacteria-killing abilities of plasma proteins, including natural antibodies and complement, as well as lysozyme and constitutively produced acute phase proteins (Matson et al., 2006; Sparkman and Palacios, 2009). In addition to the measures of constitutive innate immunity, acquired immunity can be measured through the levels of peripheral

lymphocytes. Lymphocytes are the major component of the acquired immune system and their abundance may be used as a measure of investment in induced acquired immunity (Lee, 2006; Sparkman and Palacios, 2009). Nutritional deficiency, and thus stress, can also be quantified through two different measures: levels of the glucocorticoid, corticosterone, and heterophil to lymphocyte ratios in peripheral blood (Vleck et al., 2000). Glucocorticoids are secreted by the adrenal cortex (Salpolsky, 1992) and typically circulate in the bloodstream at baseline levels, regulating critical metabolic processes such as energy acquisition, storage, and utilization (Sapolky et al, 2000; Landys et al., 2006; Palacios et al., 2012). Events such as periods of food restriction however, can cause increased secretion of corticosterone which can cause a shift in physiology and behavior such that self-maintenance and survival processes are prioritized, many times at the expense of less immediately vital functions (Palacios et al., 2012). Alternatively, the ratio of heterophil to lymphocytes is also an indicator of stress levels (Gross and Siegel 1983; Maxwell, 1993). Long-term stressors, such as food deprivation, elevate the number of heterophils and depress the number of lymphocytes in the blood and thus can be utilized as a supplement or replacement to circulating levels of corticosterone as leukocyte numbers change more slowly in response to stress than does corticosterone (Gross and Siegel, 1986; McFarlane and Curtis, 1989).

SEXUAL SIZE DIMORPHISM

A sex difference in size and body proportion is known as sexual size dimorphism (Shine and Crews, 1988; Butler, 2007). Dimorphism in adult size typically occurs due to differential growth rates between sexes or because of sexual maturity being reached at different ages. Body size is one of the most important quantitative traits of an organism

because of its ubiquitous effects on physiological, ecological and life-history processes (John-Adler et al., 2007). Interspecific differences in adult body size are thought to reflect selection for niche diversification (Butler, 2007). Intraspecific differences between sexes (sexual size dimorphism) are typically interpreted as evidence for natural and sexual selection for a reproductive advantage in both male and female body size (Andersson, 1994; John-Adler et al., 2007). Large body size for males is advantageous in agonistic encounters where size can determine access to potential mates and thus reproductive success (Shine, 1994; Cox et al., 2003; 2007). In species that exhibit variable clutch sizes, a larger size in females has a reproductive advantage because the number of eggs, offspring or size of offspring increases with body size (John-Adler et al., 2007). Thus, it is predicted that sexual selection should favor large size in males, while fecundity selection should favor large size in females (Cox et al. 2007; John-Adler et al., 2007).

SNAKES AS A STUDY ANIMAL

Snakes are highly relevant models for behavioral, ecological or physiological studies due to a variety of factors. Snakes are a diverse group with over 2500 species described (Webb et al., 2000) in which parental provisioning is rarely seen, guarding of eggs occurs in only about 3% of squamate (snakes and lizards) genera (Shine, 1988), thus neonates can be obtained from birth/hatching without negative effects. More specifically, snakes are relevant models for studying compensatory growth because they play important roles in ecosystems as predators and occupy a wide range of niches and habitats. Resource, and thus energy, intake of snakes is more easily calculated than in other animals (Madsen and Shine, 2000; 2002) since food intake does not need to be

estimated because snakes consume their prey whole. In addition, snakes are evolutionarily adapted to cope with variation in prey availability and their adult body size is strongly dependent on energy intake during the pre-adult life stages (Madsen and Shine, 2000). Snakes respond to abundant food with rapid growth but can tolerate low levels of energy intake for long periods without apparent ill effects on overall health (Ford and Seigel, 1994). However, as for other organisms, the nutritional conditions an individual experience during early development may impact their overall growth trajectory.

The checkered garter snake, *Thamnophis marcianus,* is a viviparous colubrid species in which males do not compete for females. In this species strong female biased sexual size dimorphism occurs. Garter snakes are the most studied snake model system in the areas of ecology, evolution, behavior and physiology (Castoe et al., 2011), the vast amount of research and knowledge about the species make them an ideal model for study. In contrast the corn snake, *Pantherophis guttata,* is an oviparous colubrid species that occurs in southeastern U.S. and exhibits strong male biased sexual size dimorphism, the males compete for access to females and larger size is a selective advantage (Ernst and Ernst, 2003).

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Although compensatory growth, and the subsequent trade-offs with different life-history traits, has been studied in a variety of organisms, there is less empirical evidence that the same patterns occur in ectothermic animals such as snakes. Ectothermic organisms such as insects (i.e. caddis flies, butterflies, beetles) and amphibians (i.e. guppies) have shown similar effects to compensatory growth as birds, fish and mammals

but longer-lived ectothermic organisms such as snakes have been less studied. Snakes not only are relatively long lived in comparison to other organisms in which this phenomenon of compensatory growth has been studied, but they also exhibit indeterminate growth and thus indeterminate size. For females, regardless of species, it is a fecundity advantage to maintain high growth rates to achieve a larger size and thus it is anticipated that compensatory growth will not occur between dietary treatment groups, regardless if the low diet group allocates more energy to growth when food becomes more abundant. For the checkered garter snake, *T.marcianus,* sexual size dimorphisms favor the female. The males in this species do not compete for access to females and thus large size is not a life-history advantage as long as reproductive size is achieved. It is suggested that the low diet treatment group for male *T.marcianus* will exhibit compensatory growth when food becomes more available; as large size is not an advantage it is expected that the high diet treatment males will slow their growth rates as they achieve mature size. In particular, exposure to predator would select for reduction in foraging activity. Contrary to the checkered garter snake, the corn snake (*P.guttata*) is a species in which males do compete for access to females and thus large size is a reproductive advantage. Compensatory growth is anticipated to not occur between the dietary treatment groups, as it is advantageous for the high diet animals to maintain a high rate of growth, making it difficult for the low diet animals to "catch-up" even if excess energy, obtained through increased resources, is allocated into growth.

The allocation of resources towards growth, whether compensatory growth occurs or not, has been shown to trade-off with other traits such as exploratory behavior (e.g. Gottard, 2000), general cognition (e.g. Fisher et al., 2006), reproduction (e.g. Inness and

Metcalfe, 2008; Auer et al., 2010) and longevity (Lee et al., 2013) if there is a greater benefit in achieving a larger size more quickly. Although it is known that levels of food and other environmental resource based factors can impact immunity in wild populations of snakes it has not been demonstrated that compensatory growth requires a downgrading of the immune response during that period or afterward. Immune function is expensive to maintain and thus could potentially be a function that is down regulated when excess energy is needed for growth (Sheldon and Verhulst, 1996; Norris and Evans, 2000; Lochmiller and Deerenberg, 2000). It is expected that adaptive immunity will trade-off with growth, as the ability to mount responses against pathogens at an adaptive level is more costly than innate immunity. Thus, we would expect to see a decrease in adaptive immune response, and potentially innate responses in low diet animals (regardless the species and sex) depending on the level of resource allocation, i.e. when high growth rates occur.

Immune function is complex, and the relative contribution of different components (e.g. cellular vs. humoral) within species during periods of either homeostasis or stress is unclear (Matson et al., 2006). Relatively little is known about the intra- or interspecific variation in immunological capabilities, especially in the context of the effects of stress on immune function (Matson et al., 2006). Upon exposure to unpredictable adverse events such as increased risk of predation, severe climatic conditions or food deprivation, the activity of the hypothalamic-pituitary-adrenal (HPA) axis (hypothalamic-pituitary-interrenal (HPI) axis in reptiles) (Greenberg and Wingfield, 1987) is up-regulated and secretion of glucocorticoids is increased (Palacios et al., 2012). Stress induced levels of glucocorticoids, corticosterone being the major glucocorticoid in

reptiles (Greenberg and Wingfield, 1987), cause a shift in physiological and behavioral parameters such that self-maintenance and survival processes are prioritized (Palacios et al., 2012). By redirecting resources and behaviors, corticosterone and thus stress especially at chronic levels is generally considered immunosuppressive (Råberg et al., 1998).

Therefore, in the snake species utilized in this experiment, it is hypothesized that the male garter snakes will exhibit the strongest compensatory growth; exhibit increased levels of corticosterone and a shift in immune function from adaptive to innate. Alternatively, similar responses may occur in all low diet treatment groups since the groups may exhibit some increase in growth rate (just can not catch-up).

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CHAPTER TWO

COMPENSATORY GROWTH IN SNAKES WITH DIFFERENT SEXUAL DIMORPHISMS

INTRODUCTION

Theoretically, an individual should match its phenotype to the current environmental conditions to maximize fitness (Roff, 2002). Though many life-history traits are relatively inflexible, extrinsic factors such as temperature, predation risk or low resource availability are likely responsible for variation in attributes such as growth rates and ultimately adult body size (Adolph and Porter, 1993, 1996; Madsen and Shine, 1996, 1999, 2000; Radder et al., 2007). Nutritional stress during natal periods, in particular, has significant long-term consequences for adult characteristics (Kubicka and Kratochvil, 2009).

Nutritional stress can be a result of insufficient time to acquire resources for those born late in a breeding season, being born into poor environmental conditions or due to poor parental provisioning in the egg or juvenile stages (Metcalfe and Monaghan, 2001). Food availability can also be unpredictable and highly variable in natural environments thus growth rates of individuals often respond accordingly (Radder et al., 2007). Even when subsequent improvement in prey availability allows for compensation for prior reduced growth, an early-restricted diet can profoundly effect adult phenotypes and fitness.

The term "compensatory growth" or "catch-up growth" describes both the pattern and the process by which an organism grows more quickly after a period of reduced growth than would be expected in the absence of restriction (Ali et al, 2003; Metcalf and Monaghan, 2001; Bjorndal et al., 2003; Nicieza and Alvarex, 2009). Growth rates are flexible and are usually regulated at optimal rather than maximal rates, such that rates can be increased when selective pressures favor an increase in overall size (Arendt, 1997; Metcalfe and Monaghan, 2001; Mangel and Munch, 2005; Stoks et al., 2006).

Poor nutritional conditions at natal stages can also affect one sex more severely than the other (Metcalfe and Monaghan, 2001; Le Galliard et al., 2005) such that typically the faster growing sex is more vulnerable to restricted food availability (Tschirren et al., 2003). These responses to natal nutritional stress are proposed to have different mechanisms, which are hypothesized to depend on whether selection favors increased size at age of first reproduction or for early reproduction regardless of size. When increased size at age of first reproduction is selected for, smaller females that would have smaller or fewer offspring are selected against. The drive to compensate for small size may also differ between the sexes because many species exhibit sexual size dimorphism in adult size; for some species females grow larger than males and in others males reach a larger adult size. In males larger size is advantageous in species that display intrasexual competition. In females size is an advantage for fecundity reasons, especially in species that exhibit indeterminate growth and indeterminate fecundity (Taylor and DeNardo, 2005). Thus, it is hypothesized that natal nutritional stress causing an inhibition in growth, followed by a period of increased resources will allow for allocation of energy towards increased growth rates. Male *Thamnophis marcianus*

(checkered garter snakes), a species in which males do not compete for females and thus a larger overall size is not an evolutionary advantage, is hypothesized to fully compensate in size when resources are plentiful and catch up to their non-stressed conspecifics. Male *Pantherophis guttata* (corn snakes) and females of both species are hypothesized to partially compensate in size; excess energy should be allocated into growth, however, it is advantageous for each to maintain a larger adult body size and thus there should be no "slowing down" of growth when a mature size is reached in the non-stressed individuals making the nutritionally stressed animals incapable of catching-up in overall size.

METHODS

STUDY ANIMALS

Animal Care and Handling

This study was conducted with 69 specimens of checkered garter snake, *Thamnophis marcianus,* and 44 specimens of corn snake, *Pantherophis guttata;* the neonates for both species came from colonies maintained by the University of Texas at Tyler's Ophidian Research Colony (ORC). All individuals were maintained following IACUC protocol #UTT-006 under Dr. Neil B. Ford. Individuals were housed separately in size-appropriate translucent plastic storage boxes, each filled with 3-5cm of aspen bedding with water available *ad libitum*. Animals were maintained on a 12:12 light/dark cycle at 27±1°C to simulate a photoperiod and temperature regime commonly experienced throughout their Texas range during the active season (Rossman et al., 1996).

Feeding & Growth

For this study male and female offspring, from females of both species, were randomly but evenly and sexually split into two dietary groupings (high and low) – this is a 2 species x 2 diets x 2 sexes fully factorial design. For the first two weeks of life the *T.marcianus* neonates were offered pieces of tadpole tail to help ensure regular eating patterns, as this species' diet in the wild consists mainly of tadpoles, frogs and fish (Rossman et al., 1996). Starting the third week of life the animals were offered thawed newborn mice that provide a higher nutritional content; these were scented with tadpole to promote feeding until the animals were adjusted to the altered diet. For the first two weeks of life the *P.guttata* neonates were offered *ad libitum* pieces of thawed newborn mice to ensure regular eating patterns. Starting the third week of life each animal was offered mice in amounts consistent with their individual mass and specific dietary regime. For both species, the animals on a high dietary regime were fed 60% of their own mass weekly over two feedings; this amount approximated *ad libitum* feeding as established by an earlier pilot test. The animals on a low dietary regime were offered 20% of their own mass in food weekly in one feeding. Growth was known to still occur and animals remain healthy. The animals were maintained on these diets until a significant separation in mass between the two test groups was achieved and the low diet animals on average doubled their birth mass. Animals were weighed (in grams) and snout-vent lengths (SVL) measured (in centimeters) at birth (*T.marcianus*) or hatching (*P.guttata*), and thereafter weighed every 15 days and SVL measured every 30 days to track separation between the groups. Once an adequate amount of time had passed to exhibit separation between the dietary groupings (105 days for *T.marcianus* and 175 days

for *P.guttata*) the low diet animals were given the opportunity for compensatory growth by raising their diet regimen to match that of the high diet animals. During this second phase of the experiment, the animals were fed twice a week, multiple food items to measure 60% of their mass between the two feedings. Multiple food items were offered to allow for the snakes to make choices in the amount of food ingested. Measurements of mass and SVL were still recorded at 15-day and 30-day intervals respectively to track whether or not the low diet animals were "catching up" in size. Allowing equal time for compensatory growth as was allotted for separation (105 days for *T.marcianus* and 175 days for *P.guttata*); growth means and rates for mass and SVL within each time period were analyzed between treatment groups for each sex.

COMPENSATORY GROWTH

Compensatory growth was assessed by comparing the treatment groups (within sexes and species) across the two phases of growth. Mean mass and SVL for each group was compared at birth and hatching for *T.marcianus* and *P.guttata* respectively, at the end of the first phase of growth, and at the end of the second phase of growth. Growth rates between the two growth phases were also analyzed between the groups, where growth rate was measured as $(G_1 - G_2)/T$ (where G is the mean growth for either mass or SVL and T is the time in days).

STATISTICAL ANALYSIS

All analyses were conducted using R 2.15.3 statistical package (R Core Team, 2013). All data were checked for normality and homoscedasticity and were transformed as necessary. Data that were non-normal after transformation were analyzed using

equivalent non-parametric tests. Data were separated by species and sex, the means between diet treatments for mass and SVL at the 3 time points (Birth/Hatching, Phase 1, and Phase 2) were analyzed using a Student's T-Test for normal data and a Mann-Whitney U test for non-normal data. Since both species exhibit sexual size dimorphisms, and differences in mass and SVL are expected (except at birth/hatching) analyses were not conducted between sexes. Growth rates between groups were analyzed using a Student's T-Test for normal data and a Mann-Whitney U test for non-normal data, while within group growth rates across phases were analyzed using a paired T-Test for normal data and a pairwise Wilcoxon test for non-normal data.

RESULTS

Female Growth (Thamnophis marcianus)

Snakes assigned to the two dietary treatment groups did not differ in mass or SVL at birth (Mann-Whitney U test, n = 38, U = 188, $P = 0.734$; Student's T-Test, n = 38, t = −0.307, $P = 0.761$, for mass and SVL respectively; Fig 1). Compared with the low diet treatment, neonates on the high diet treatment grew at a faster rate for both mass and SVL during the first growth phase (Mann-Whitney U test, n = 38, U = 352, $P < 0.0001$ for the increment in mass; n = 38, U = 352, $P < 0.0001$ for the increment in SVL; Fig 1) and thus at the end of the first phase of growth (birth – day 105) averaged significantly larger in size (Mann-Whitney U test, n = 38, U = 352, $P < 0.0001$; n = 38, U = 352, $P < 0.0001$, for mass and SVL respectively; Fig 1). During the second phase of growth (day 105 – day 210), where both treatment groups were offered 60% of their mass weekly in multiple feedings to simulate *ad libitum* feeding, both dietary treatment groups exhibited a significant increase in growth rate (Wilcoxon, n = 16, W = 0, $P < 0.0001$; n = 22, W =

0, $P < 0.0001$ for measurements of mass in high diet and low diet treatment groups respectively; n = 16, W = 26, $P = 0.029$; n = 22, W = 0, $P < 0.0001$ for measurements of SVL in high diet and low diet treatment groups respectively; Fig 1). As in the first phase of growth, the high diet treatment group maintained a higher rate of growth for mass than the low diet treatment group (Student's T-Test, n = 38, t = 3.206, $P = 0.004$; Fig 1) but the measurement of growth for SVL showed indistinguishable rates (Student's T-Test, n = 38, t = 0.215, $P = 0.832$; Fig 1). Thus at the end of the second phase of growth the high diet treatment group remained on average larger than the low diet treatment (Mann-Whitney U test, n = 38, U = 311, $P < 0.0001$; Student's T-Test, n = 38, t = 5.209, $P < 0.0001$, for mass and SVL respectively; Fig 1).

Male Growth (Thamnophis marcianus)

Snakes assigned to the two dietary treatment groups did not differ in mass or SVL at birth (Student's T-Test, n = 31, t = -0.407, $P = 0.688$; Student's T-Test, n = 38, t = −0.334, $P = 0.741$, for mass and SVL respectively; Fig. 2). Compared with the low diet treatment, neonates on the high diet treatment grew at a faster rate for both mass and SVL during the first growth phase (Mann-Whitney U test, n = 31, U = 238, $P < 0.0001$ for the increment in mass; n = 31, U = 237, $P < 0.0001$ for the increment in SVL; Fig 2) and thus at the end of the first phase of growth (birth – day 105) averaged significantly larger in size (Mann-Whitney U test, n = 31, U = 233, $P < 0.0001$; Student's T-Test n = 31, t = 6.027, $P < 0.0001$, for mass and SVL respectively; Fig 2). During the second phase of growth (day 105 – day 210) the high treatment group did not exhibit a significant change in growth rate for mass but did for SVL from the first growth phase (Wilcoxon, n = 16, W = 34, $P = 0.083$; n = 16, W = 108, $P = 0.039$ for mass and SVL respectively; Fig 2)

while the low diet treatment group exhibited a significant increase in growth across both measurements (Paired T-Test, n = 15, t = -9.106, $P < 0.0001$; n = 15, t = -7.249, $P < 0.0001$ for mass and SVL respectively; Fig 2). Growth rates between treatment groups showed the reverse pattern to that seen during the first growth phase. The low treatment group grew more rapidly during this phase than the high diet treatment group (Mann-Whitney U test, n = 31, U = 72, $P = 0.059$; Student's T-Test, n =31, t = −2.886, $P = 0.008$ for mass and SVL respectively; Fig 2). Thus mean body measurements were indistinguishable at the end of the second phase of growth between the two dietary treatment groups (Mann-Whitney U test, n = 31, U = 128, $P = .770$; Student's T-Test, n = 31, t = 1.392, $P = 0.175$, for mass and SVL respectively; Fig 2).

Female Growth (Pantherophis guttata)

Snakes assigned to the two dietary treatment groups differed slightly in mass but not SVL at the beginning of the study, however this was not deemed biologically significant as the animals were assigned groups randomly and the difference was very low relative to the increase in mass over the length of the experiment (Student's T-Test, n = 16, t = 2.358, $P = 0.035$; n = 16, t = 1.952, $P = 0.078$, for mass and SVL respectively; Fig. 3). Compared with the low diet treatment, neonates on the high diet treatment grew at a faster rate for both mass and SVL during the first growth phase (Mann-Whitney U test, n = 16, U = 64, $P = 0.0009$ for the increment in mass; n = 16, U = 64, $P = 0.0009$ for the increment in SVL; Fig 3) and thus at the end of the first phase of growth (day 0 – day 175) averaged significantly larger in size (Mann-Whitney U test, n = 16, U = 0.85, $P = 0.0009$; n = 16, U = 64, $P = 0.0009$, for mass and SVL respectively; Fig 3). During the second phase of growth (day 175 – day 350), both dietary treatment groups exhibited a

significant increase in growth rate (Paired T-Test, n = 8, t = -5.591, $P = 0.0008$; n = 8, t = -10.564, $P < 0.0001$ for measurements of mass in high diet and low diet treatment groups respectively; n = 8, t = 0.621, $P = 0.555$; n = 8, t = -12.014, $P < 0.0001$ for measurements of SVL in high diet and low diet treatment groups respectively; Fig 3). As in the first phase of growth, the high diet group maintained a higher rate of growth for mass than the low diet group (Mann-Whitney U test, n = 16, U = 0.86, $P = 0.028$; Fig 3) but the measurement of growth for SVL showed the opposite pattern with the rate of growth for the low diet group surpassing that of the high diet group (Student's T-Test, n = 16, t = -2.785, $P = 0.016$; Fig 3). However, despite increased rates of growth, at the end of the second phase of growth the high diet treatment group remained on average larger than the low diet treatment (Student's T-Test, n = 16, t = 4.039, $P = 0.003$; n = 16, t = 4.211, $P = 0.0008$, for mass and SVL respectively; Fig 3).

Male Growth (Pantherophis guttata)

Snakes assigned to the two dietary treatment groups did not differ in mass or SVL at the beginning of the study (Student's T-Test, n = 28, t = 1.986, $P = 0.058$; n = 28, t = 0.499, $P = 0.623$, for mass and SVL respectively; Fig. 4). Compared with the low diet treatment, neonates on the high diet treatment grew at a faster rate for both mass and SVL during the first growth phase (Mann-Whitney U test, n = 28, U = 196, $P < 0.0001$ for the increment in mass; n = 28, U = 8.25, $P < 0.0001$ for the increment in SVL; Fig 4) and thus at the end of the first phase of growth (day 0 – day 175) averaged significantly larger in size (Mann-Whitney U test, n = 28, U = 196, $P < 0.0001$; n = 28, U = 10.84, $P = < 0.0001$, for mass and SVL respectively; Fig 4). During the second phase of growth (day 175 – day 350), both dietary treatment groups exhibited a significant increase in growth

rate for mass (Paired T-Test, n = 14, t = -8.391, $P < 0.0001$; n = 14, t = -21.786, $P < 0.0001$ for high diet and low diet treatment groups respectively; Fig 4) however only the low diet treatment group demonstrated an increase in growth rate for the measure of SVL (Paired T-Test, n = 14, t = -1.338, $P = 0.204$; n = 14, t = -14.396, $P < 0.0001$ for high diet and low diet treatment groups respectively; Fig 4). As in the first phase of growth, the high diet treatment group maintained a higher rate of growth for mass than the low diet treatment group (Mann-Whitney U test, n = 28, U = 0.16, $P = 0.004$; Fig 4) while the rate of growth for SVL was indistinguishable between the two treatment groups (Student's T-Test, n = 28, t = -1.706, $P = 0.104$; Fig 4). Thus at the end of the second phase of growth the high diet treatment group remained on average larger than the low diet treatment group (Mann-Whitney U test, n = 28, U = 185, $P < 0.0001$; Student's T-Test, n = 28, t = 4.206, $P = 0.0003$, for mass and SVL respectively; Fig 4).

DISCUSSION

These data demonstrate the role that natal nutrition has on the growth rates of individuals in two species of snake that differ in sexual size dimorphism. It was hypothesized that animals stressed with low food availability during natal periods would compensate for size deficiency by allocating energy in subsequent periods, when food was abundant, into rapid growth if large size in itself was a life-history advantage. However, if large size is a reproductive advantage as for fecundity in females, or in males in which agonistic encounters determine reproductive success, then allocating energy into growth may not result in complete size compensation as it would be beneficial to the non-dietary stressed conspecifics to maintain high rates of growth. Therefore this effect was

hypothesized to occur only in the males of the species that does not exhibit agonistic behaviors (i.e. checkered garter snakes).

Female Growth (Thamnophis marcianus)

As hypothesized, compensatory growth did not occur in the low diet treatment group for female *T.marcianus.* Animals raised on high and low dietary regimes showed differences in both mass ($P < .0001$) and SVL ($P < .0001$) at the end of the first growth phase. During the second growth phase, when food was offered *ad libitum* to all animals, both dietary treatments groups increased their rate of growth for mass ($P < .0001$ for both high and low diet treatments) and SVL ($P = .029$; $P < .0001$ for high and low diet treatment groups respectively). Thus at the end of the second growth phase there remained a difference in mass ($P < .0001$) and SVL ($P < .0001$) with the high diet treatment group maintaining a larger size in both growth components.

Though compensatory growth did not occur between the dietary treatment groups for female *T.marcianus,* the low diet group increased its growth rate, for SVL, to match that ($P = .831$) of the high diet treatment group. The increase in rate of growth by the low diet treatment group to match that of the high diet group suggests that the low diet group reached the maximum growth rate while the high diet animals were maintaining maximal rates. This suggests that potentially the measurement of length is more important evolutionarily than is mass when one growth component needs to be conserved over the other. Mass can be related to energy storage, which is beneficial in times of food restriction though potentially not as important in reproduction. The trend of increased rate in SVL of the low diet regime to match or surpass that of the high diet group suggests that perhaps with a longer time period the low diet treatment would

compensate for size in length. Body size is one of the most important quantitative traits of an organism because of its ubiquitous effects on physiological, ecological and life-history processes (John-Adler et al. 2007) and thus we would expect that, if possible, females would increase in size. A larger size in females has a reproductive advantage because the number of eggs, offspring or size of offspring increases with body size in species, such as garter snakes, that exhibit variable clutch sizes (John-Adler et al. 2007).

Male Growth (Thamnophis marcianus)

As hypothesized, compensatory growth did occur in the low diet treatment group for male *T.marcianus.* Animals raised on high and low dietary regimes showed differences in both mass ($P < .0001$) and SVL ($P < .0001$) at the end of the first growth phase. During the second growth phase, when food was offered *ad libitum* to all animals, the high diet treatment group did not increase its rate of growth for mass ($P = .083$) but did for SVL ($P = .039$). However, the low diet treatment group increased both its rate for mass ($P < .0001$) and SVL ($P < .0001$) to the point where both measurements surpassed that of the high diet treatment ($P = .059$; $P = .007$ for mass and SVL respectively). Thus, at the end of the second growth phase there was no difference between the two dietary treatment groups for either mass ($P = .770$) or SVL ($P = .175$). The ability of the low diet treatment animals to compensate for both mass and SVL was as hypothesized. Large size is not a reproductive advantage for the males in this species, as they do not exhibit agonistic behaviors, and thus continuing to grow past a mature size does not offer any life-history advantages. High diet males did not increase mean rates of growth and thus this allows for compensation to occur in the low diet group by increasing mean growth rates to surpass that of their non-stressed conspecifics.

Female Growth (Pantherophis guttata)

As hypothesized, compensatory growth did not occur in the low diet treatment group for female *P.guttata.* At the end of the first growth phase, animals raised on high and low dietary treatment regimes showed differences in both mass ($P = .0009$) and SVL ($P = .0009$). During the second growth phase the high diet treatment group increased its rate of growth for mass ($P = .0008$) but decreased its rate of growth for SVL, though not by a significant margin ($P = .555$). In comparison, the low diet treatment group increased its rate of growth for both mass ($P < .0001$) and SVL ($P < .0001$) with its rate of growth for SVL surpassing the rate of the high diet treatment group ($P = .016$). However, at the end of the second growth phase the high diet treatment group maintained a larger size for both mass ($P = .003$) and SVL ($P = .0009$).

Though compensatory growth did not occur between the dietary treatment groups for female *P.guttata,* the low diet treatment group increased its growth in SVL to surpass that of the high diet treatment group, suggesting that with an extended time period compensatory growth would occur for the growth measurement in length. High diet females should continue to grow as much as possible since size relates to increased fecundity. Only at maturity should the energy shift into reproductive output. A catch-up in length could potentially signify the importance of length over mass for reproductive output (as suggested for female checkered garter snakes); however, a caveat to this may be that mass is not the leading factor in reproductive capability but perhaps the driving force in determining clutch size or offspring size.

Male Growth (Pantherophis guttata)

As hypothesized, compensatory growth did not occur in the low diet treatment group for male *P.guttata.* High diet males would be expected to continue to grow at maximal rates since size relates to success in mating for this species. At the end of the first growth phase, animals raised on high and low dietary regimes showed differences in both mass ($P < .0001$) and SVL ($P < .0001$). During the second growth phase both treatment groups increased their rate of growth for mass ($P < .0001$ for both treatment groups) while only the low diet treatment group increased its rate of growth for SVL ($P = .204$; $P < .0001$ for high and low diet treatment groups respectively). The low diet treatment groups' rate of growth for SVL matched the rate of the high diet group ($P = .104$). However, because of their high growth rate, the high diet treatment groups still maintained a larger size in both mass ($P < .0001$) and SVL ($P = .0003$) at the end of the second growth phase.

Though compensatory growth did not occur between the dietary treatment groups for male *P.guttata,* the increase in growth rate of SVL for the low diet treatment group potentially suggests that overall size in length may be more important than an increase in mass in reproductive success when competing for females. The growth rate for SVL, though not significant, was higher during the second growth phase for the low diet treatment group suggesting that perhaps with an extended time period allowed, compensation would occur in the growth measurement of length. Though maintaining a high growth rate is important in high diet male *P.guttata*, for reproductive purposes, a trend towards catching up in size by the low diet animals may be occurring due to the long life span of the species. Animals that are longer lived are likely to have multiple

opportunities to reproduce which suggests that reaching a larger size, even if they must reproduce at a later time point (i.e. miss first reproductive opportunity) would be a better strategy than maintaining a smaller size (Ford and Seigel, 1994). Reaching a larger size would allow the animal to increase the number of offspring it can produce and thus its overall reproductive output, which would allow them to catch up in future mating events (Ford and Seigel, 1994).

Conclusions

Though compensatory growth did not occur for females in either species or for male *P.guttata,* rates of growth for the low diet treatment groups increased by margins that may imply that the animals increased their growth rates from optimal levels to try and compensate for at least partial size deficiencies. A departure from optimal growth rates would necessitate allocation of more energy resources into growth and thus cause a trade-off elsewhere. Alternatively, since large size is a life-history advantage for these groups the high diet animals may have sustained high growth rates as an effect of the 'silver-spoon effect' (Grafen, 1988); the hypothesis that food availability early in an organism's life can allow them to grow faster later in life than smaller individuals in the cohort. This can have a disproportionate effect on its later growth pattern and eventual maximum body size (Madsen and Shine, 2000). Another possibility is that the selective pressures for reproduction are not strong enough to produce a substantial shift in growth for snake species. Growing slowly may not have as large a consequence as factors involved in obtaining more prey or shifting energy allocation. For example, predation risk might increase with additional foraging time. Compared to other organisms in which compensatory growth studies have been done (e.g., Radder et al., 2007; Inness and

Metcalfe, 2008; Lee et al., 2013), snakes are long-lived animals. Having a long lifespan allows more time to reach reproductive maturity and with lower predation rates, potentially decreasing the pressures to reach a mature size at a younger age.

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TABLES & FIGURES

Table 1. Mean growth measurements and growth rates for female *Thamnophis marcianus*

	Thamnophis marcianus Female Avg. Growth Data											
	High Diet Mass (g)			Low Diet Mass (g)			High Diet SVL (cm)			Low Diet SVL (cm)		
	avg. mass	min-max	n	avg. mass	min-max	n	avg. SVL	min-max	n	avg. SVL	min-max	n
Birth	2.74	1.69-4.61	16	2.64	1.36-4.71	22	15.24	12.7-16.9	16	15.37	11.5-17.9	22
End Phase 1 (day 105)	24.15	13.19-36.24	16	6.03	2.87-8.57	22	30.96	25.7-35.1	16	21.73	16.8-24.5	22
End Phase 2 (day 210)	83.81	29.23-129.66	16	41.48	11.80-80.53	22	49.49	38.1-57.1	16	39.93	29.6-51.3	22
Rate per day Phase 1	.204	.106-.309	16	.032	.012-.056	22	.150	.111-.174	16	.061	.043-.087	22
Rate per day Phase 2	.568	.153-.938	16	.338	.068-.685	22	.177	.100-.234	16	.173	.067-.263	22

Table 2. Mean growth measurements and growth rates for male *Thamnophis marcianus*

	Thamnophis marcianus Male Avg. Growth Data											
	High Diet Mass (g)			Low Diet Mass (g)			High Diet SVL (cm)			Low Diet SVL (cm)		
	avg. mass	min-max	n	avg. mass	min-max	n	avg. SVL	min-max	n	avg. SVL	min-max	n
Birth	2.74	1.56-4.33	16	2.85	1.92-4.55	15	15.71	13.7-18.1	16	15.87	13.6-18.5	15
End Phase 1 (day 105)	16.79	6.98-29.09	16	6.35	3.42-9.85	15	29.49	22.6-35.6	16	22.42	18.6-27.3	15
End Phase 2 (day 210)	34.74	16.39-78.44	16	29.44	15.20-44.82	15	40.09	30.9-50.6	16	37.62	30.6-48.2	15
Rate per day Phase 1	.134	.045-.244	16	.033	.014-.052	15	.131	.073-.170	16	.062	.033-.083	15
Rate per day Phase 2	.171	.063-.482	16	.220	.103-.355	15	.101	.039-.210	16	.145	.083-.244	15

Table 3. Mean growth measurements and growth rates for female *Pantherophis guttata*

	Pantherophis guttata Female Avg. Growth Data											
	High Diet Mass (g)			Low Diet Mass (g)			High Diet SVL (cm)			Low Diet SVL (cm)		
	avg. mass	min-max	n	avg. mass	min-max	n	avg. SVL	min-max	n	avg. SVL	min-max	n
Start (day 0)	6.26	5.8-7.2	8	5.63	4.7-6.5	8	28.44	28.0-30.0	8	27.25	26.0-28.5	8
End Phase 1 (day 175)	39.13	31.6-46.2	8	15.41	11.3-19.3	8	51.50	50.0-54.0	8	39.31	35.5-41.5	8
End Phase 2 (day 350)	143.46	98.4-213.0	8	86.85	68.3-120.0	8	73.75	68.0-78.0	8	65.94	60.5-72.0	8
Rate per day Phase 1	.188	.144-.227	8	.056	.038-.079	8	.132	.123-.143	8	.069	.049-.086	8
Rate per day Phase 2	.596	.341-.953	8	.408	.315-.602	8	.127	.094-.157	8	.152	.137-.183	8

Table 4. Mean growth measurements and growth rates for male *Pantherophis guttata*

	Pantherophis guttata Male Avg. Growth Data											
	High Diet Mass (g)			Low Diet Mass (g)			High Diet SVL (cm)			Low Diet SVL (cm)		
	avg. mass	min-max	n	avg. mass	min-max	n	avg. SVL	min-max	n	avg. SVL	min-max	n
Start (day 0)	6.23	5.0-.78	12	5.58	4.0-6.6	14	27.96	24.5-31.5	12	27.61	25.5-30.0	14
End Phase 1 (day 175)	37.31	28.9-46.3	12	17.39	12.9-19.1	14	49.89	46.0-53.0	12	40.61	36.0-43.0	14
End Phase 2 (day 350)	144.18	96.0-197.0	12	96.03	68.0-123.5	14	74.07	66.0-81.5	12	67.46	63.5-73.0	14
Rate per day Phase 1	.178	.124-.234	12	.068	.040-.083	14	.125	.103-.171	12	.074	.049-.094	14
Rate per day Phase 2	.611	.355-.929	12	.450	.315-.606	14	.138	.094-.189	12	.154	.126-.180	14

FIGURE LEGENDS

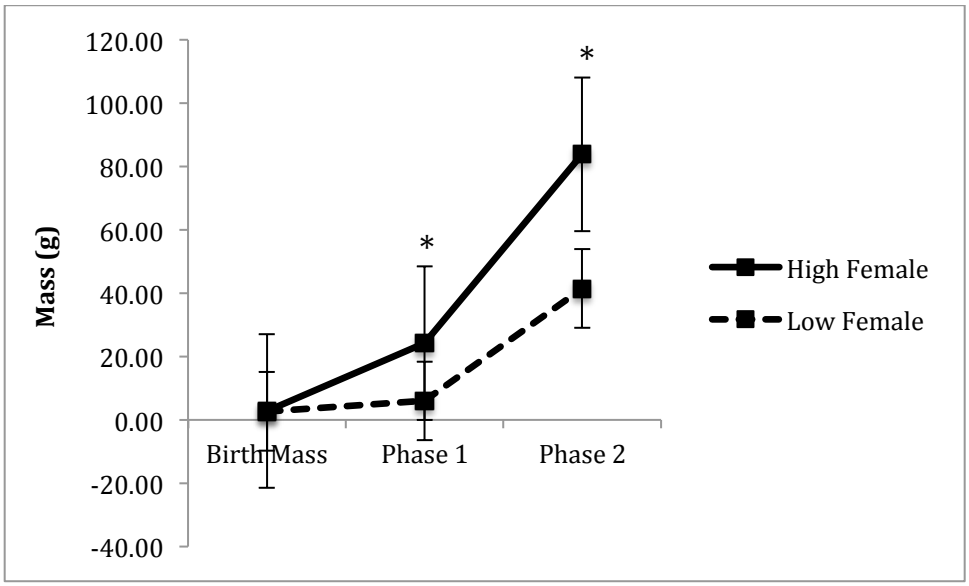
Figure 1. Effect of natal dietary restriction on the phenotypic traits of female juvenile *Thamnophis marcianus*. Graphs depict changes in (a) mass and (b) snout-vent length (SVL) across the two growth phases. Asterisks signify significant differences in mean size between the two treatment groups.

Figure 2. Effect of natal dietary restriction on the phenotypic traits of male juvenile *Thamnophis marcianus*. Graphs depict changes in (a) mass and (b) snout-vent length (SVL) across the two growth phases. Asterisks signify significant differences in mean size between the two treatment groups.

Figure 3. Effect of natal dietary restriction on the phenotypic traits of female juvenile *Pantherophis guttata*. Graphs depict changes in (a) mass and (b) snout-vent length (SVL) across the two growth phases. Asterisks signify significant differences in mean size between the two treatment groups.

Figure 4. Effect of natal dietary restriction on the phenotypic traits of male juvenile *Pantherophis guttata*. Graphs depict changes in (a) mass and (b) snout-vent length (SVL) across the two growth phases. Asterisks signify significant differences in mean size between the two treatment groups.

Figure 1



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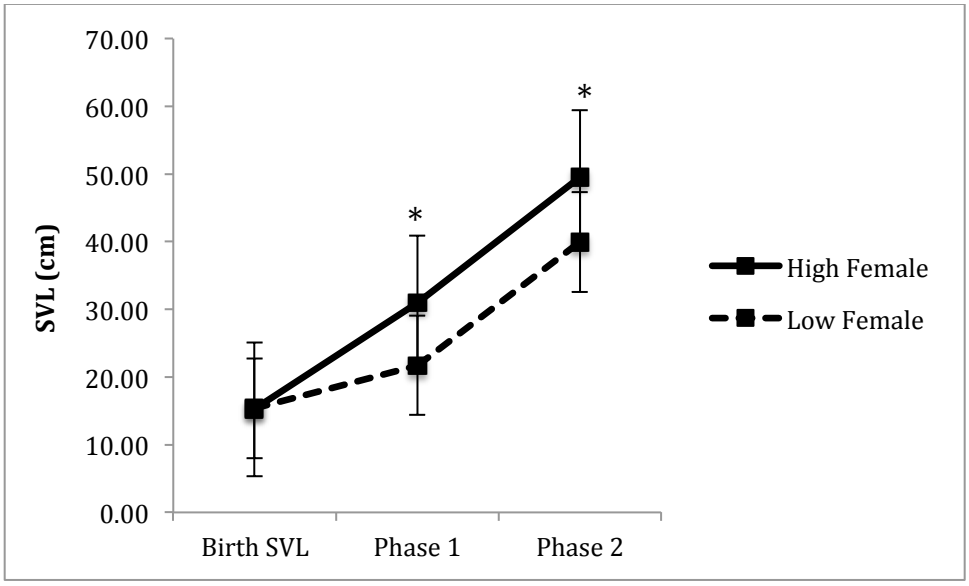
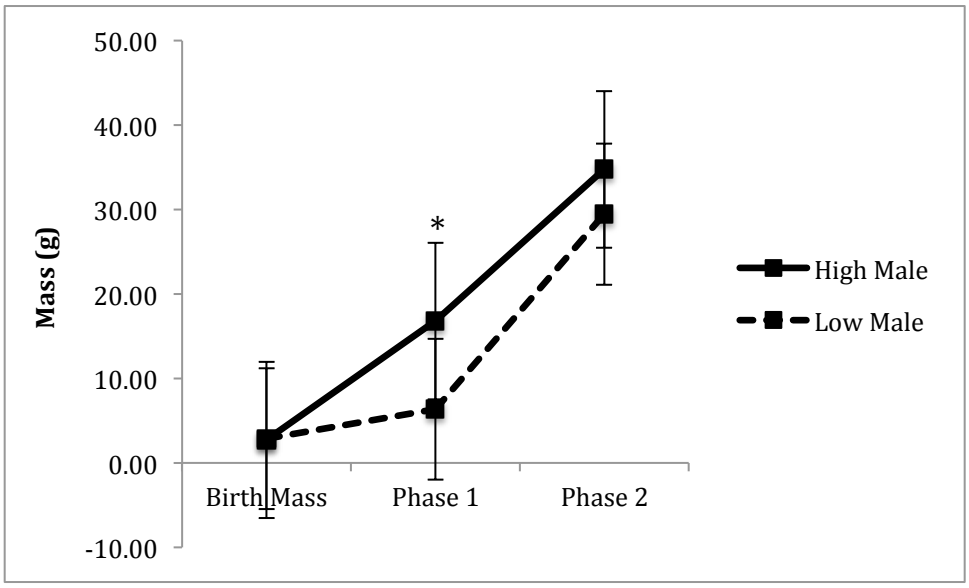


(b) *T.marcianus*

Figure 2



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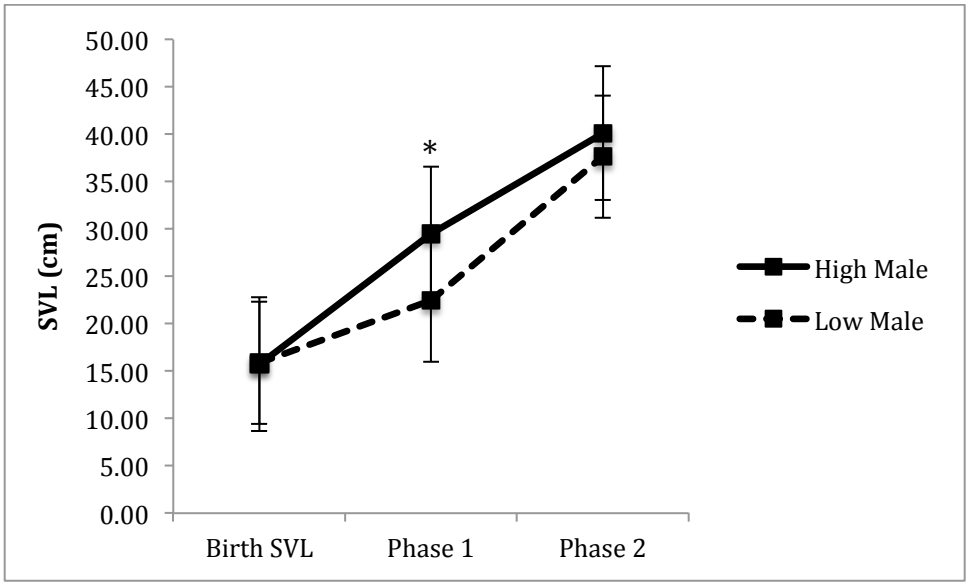

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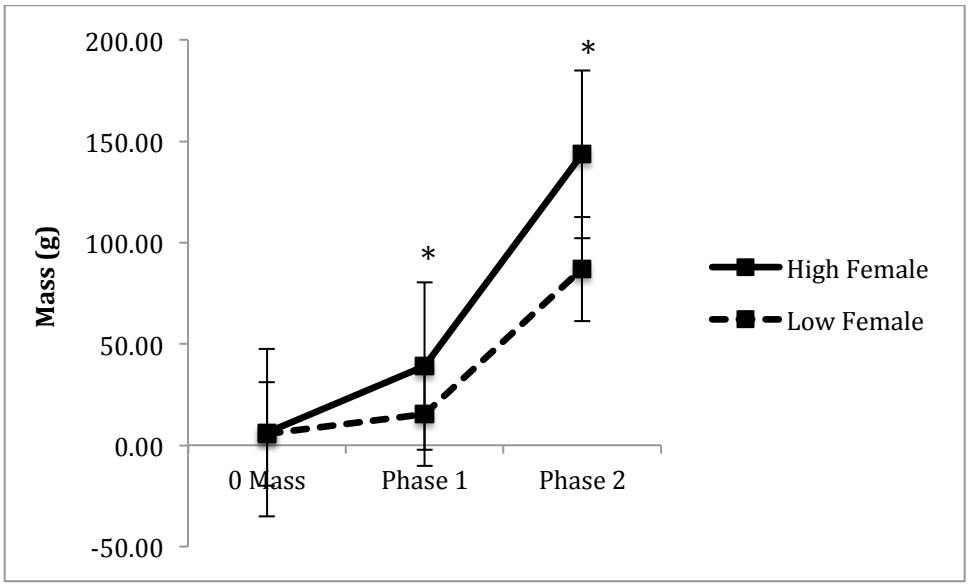
Figure 3



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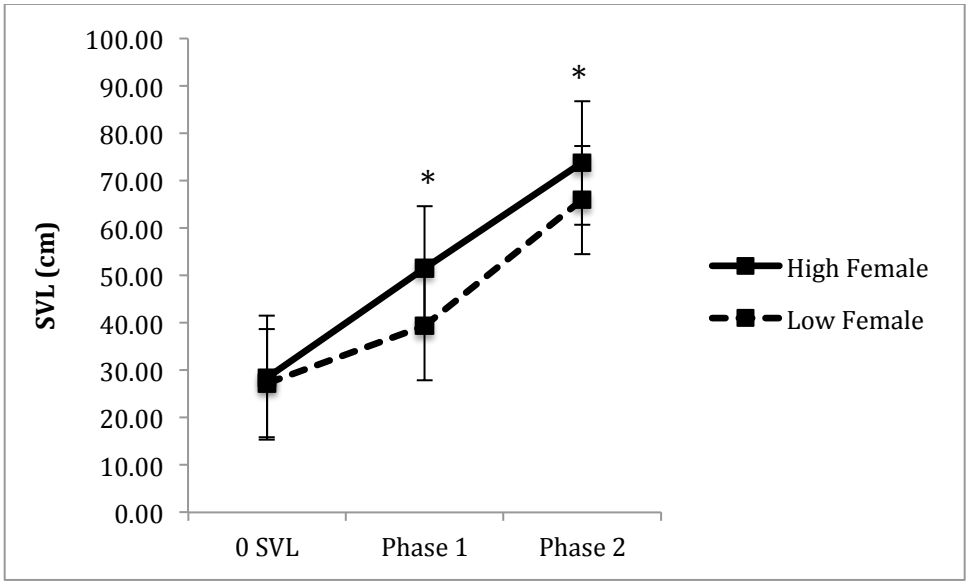
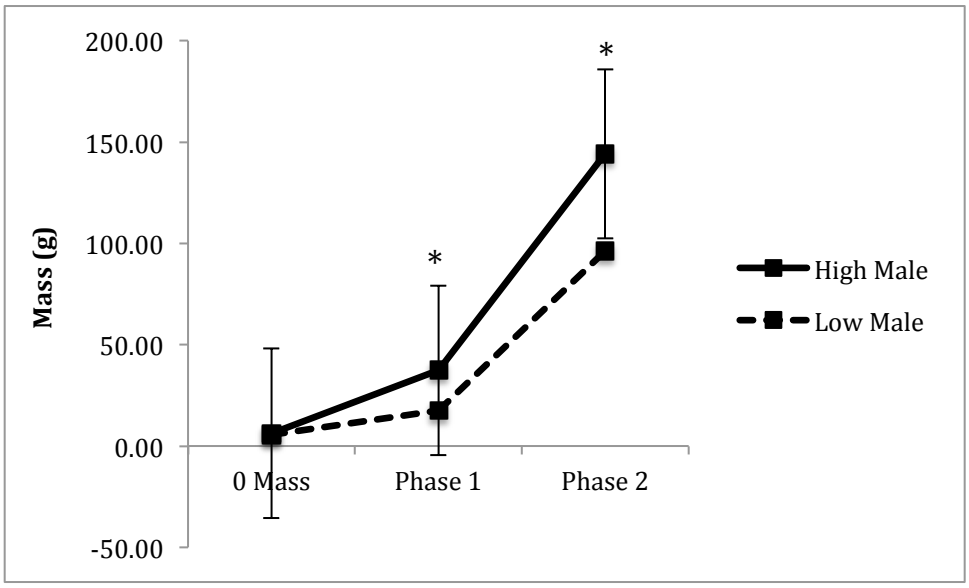


(b) *P.guttata*

Figure 4



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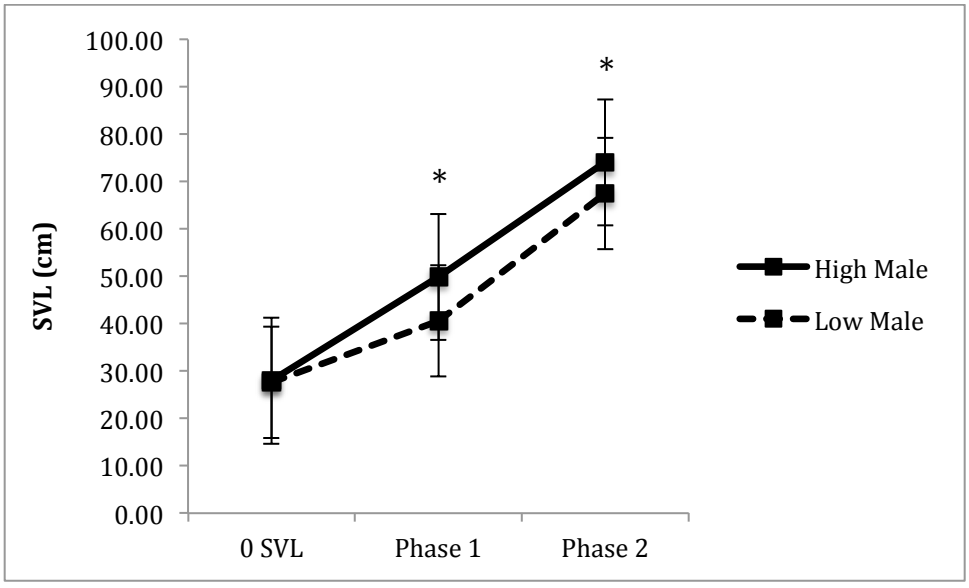

(b) *P.guttata*

CHAPTER THREE

THE ROLE OF IMMUNOLOGY IN COMPENSATORY GROWTH

INTRODUCTION

Compensatory growth is a phase of accelerated growth that occurs following a period of growth inhibition (Ali et al, 2003; Metcalf and Monaghan, 2001). This period of growth inhibition is often attributed to nutritional stress. Nutritional stress during natal periods, in particular, has been suggested to have significant long-term consequences for an organism's adult characteristics (Kubicka and Kratochvil, 2009). Compensatory responses to growth restriction have been suggested to carry physiological, developmental, ecological and immunological costs (Gotthard, 2001). Mounting an immune response and maintaining a competent immune system is a resource-demanding process that can be interchanged with growth, thermoregulation and reproduction when energy intake is reduced (Sheldon and Verhulst, 1996; Norris and Evans, 2000; Lochmiller and Deerenberg, 2000). During periods of rapid growth such as during compensatory growth, it has been predicted that innate immune constituents should prevail over induced acquired defenses, as it is less expensive to maintain than induced acquired defenses.

Early ecoimmunological interpretations of the trade-offs between resource-demanding traits, from which life-history theory is based, proposed that high investment

in growth or reproduction would result in reduced investment in immune function; causing a trade-off with self-maintenance (Sheldon and Verhulst, 1996; Norris and Evans, 2000; Schmid-Hempel, 2003). The traits of maintaining a functioning immune system and actually utilizing it are not easily quantified due to the integrated characteristics of the immune system with other physiological systems. Thus, actual attempts to exhibit the magnitude of cost of immunity have been limited. However, existing studies demonstrate that considerable nutritional costs are associated with the up-regulation of the immune system (Lochmiller and Deerenberg, 2000). Thus it is hypothesized that increased growth rates to compensate for an early growth deficiency will cause a downgrading of immune function. Constitutive adaptive immune components should more readily trade-off with growth over constitutive innate components, however, it is expected that the amount of resource allocation necessary to cause compensation (either full or partial) should down-regulate all immune constituents.

METHODS

At the end of the first phase of growth (time of the diet shift), blood samples were taken from each animal (both species and dietary groups) to test for immunological differences based on diet restriction. Blood was drawn a second time from each animal, allowing equal time for compensatory growth as was allotted for initial separation (105 days for *T.marcianus* and 175 days for *P.guttata*) and also tested for the same physiological differences. Blood was sampled to test for natural antibody and complement-mediated lysis, and bactericidal competence. Additionally, blood smears were prepared for each animal from freshly drawn blood. Blood samples of 50-250µl, depending on snake body size, were taken from the caudal vein of each animal using

heparin-rinsed syringes. Handling time to time of completion of bleeding was recorded. After centrifugation, plasma was snap-frozen in liquid nitrogen and stored in an -80 freezer.

Natural Antibodies & Complement Mediated Lysis

Natural antibodies and complement-mediated lysis were measured using a hemolysis-hemagglutination assay, as described by Matson, Ricklefs and Klasing (2005), with minor modifications. Serial two-fold dilutions of 10µl of plasma were made with PBS in a 96-well plate. Each well then received 10µl of a 2% heterologous sheep's red blood cell (SRBC) suspension. All samples were run in duplicate with positive (anti-SRBC) and negative (SRBC with no plasma sample) controls in each plate. Plates were incubated for 90 minutes at 28°C and then scored. Titers were estimated as the negative $\log_2$ of the highest dilution factor of plasma that showed hemagglutination/lysis. Half scores were given for titers that appeared intermediate. Agglutination and lysis of sheep red blood cells was utilized as this type of blood has been previously used successfully in snake/reptile studies (e.g., Kawaguchi et. al, 1978; Sparkman and Palacios, 2009).

Bacterial Competence of Plasma

Bactericidal competence, a parameter of constitutive innate immunity, was assessed according to the method described by Matson, Tieleman and Klasing (2006), with minor modifications. A pellet of lyphophilized *Escherichia coli* (Microbiologics, Cat# 0483E7) was reconstituted using 40ml of phosphate buffered saline (PBS). This was further diluted with PBS to produce working solutions that yielded roughly 200 colony-forming bacteria per 10µl. Plasma samples were diluted 1:10 with PBS. Sample

reactions were prepared by adding 10µl bacterial working solution to 100µl of the diluted plasma samples. Replicate controls were prepared (one control per every 12 samples) by adding 10µl of the bacterial working solution to 100µl of PBS. Sample reactions were incubated for 20 minutes at 28°C, the temperature the animals were maintained in the colony, to provide adequate time for bacterial killing to occur. Duplicate controls and sample reactions were plated in 50µl aliquots on 4% tryptic soy agar and incubate approximately 24 hours at 28°C, additional incubation at 37°C was used if bacterial colonies were not clearly visible. The number of bacterial colonies on each plate was counted and the percentage of colonies on each plate relative to the mean number of colonies in the control plates was calculated. This percentage was subtracted from 100 to obtain the percentage of bacteria killed.

Leukocyte abundance & heterophil/leukocyte percentage

Freshly drawn blood from each animal was fixed in methanol and stained with Wright-Giemsa stain (Fisher Scientific, Cat#SDWG80) to determine leukocyte abundances and heterophil/leukocyte percentages. The number and type of leukocytes in the snake blood was quantified by scanning the blood smears under 1000x magnification and classifying the first 100 leukocytes encountered as either lymphocytes, heterophils, eosinophils, basophils or monocytes (Strik et al., 2007). If a heterophil was not viewed within classification of the first 100 leukocytes, the count was extended until either a heterophil was viewed or 200 leukocytes were encountered.

Statistical Analysis

All analyses were conducted using R 2.15.3 statistical package (R Core Team,

2013). All data were checked for normality and homoscedasticity and were transformed as necessary. Data that were non-normal after transformation were analyzed using equivalent non-parametric tests. Data were separated by species and sex and analyzed for the effects of dietary treatment group and growth phase (representative of time and sample, first blood sample collected on either day 105 or day 175 for *T.marcianus* and *P.guttata* respectively and the second blood sample collected on either day 210 or day 350 for *T.marcianus* and *P.guttata* respectively).

The relationship between natural antibody and lysis was assessed using a simple correlation; correlated variables are typically best analyzed jointly. Since measures proved to be very strongly correlated ($r \geq .88$, see RESULTS) and thus multicollinearity became an issue for redundancy and a loss of degrees of freedom, each measure was analyzed independently with a Two-way ANOVA. Bactericidal competence was non-normal and non-homoscedastic even with reflection and transformation and thus was analyzed with a Mann-Whitney U test for between group analysis and a paired Wilcoxon test for within group analysis. Lymphocyte abundance and heterophil/lymphocyte ratios were analyzed using a Two-way ANOVA.

RESULTS

Natural Antibodies & Complement Mediated Lysis

There was a strong positive correlation between natural antibody and complement-mediated lysis titers in female *T.marcianus* (Correlation, $r = 0.95$, $df = 73$, $P < 0.0001$; Fig. 1a). The two-way ANOVA determined that overall there was significance between diet and growth phase on the mean number of agglutination titers ($F_{1, 74} = 4.845$, $P = 0.031$) and mean lysis titers and growth phases ($F_{1, 74} = 4.845$, $P = 0.044$). At the end

of the first growth phase (day 105) snakes assigned to the two dietary treatment groups exhibited no difference in natural antibody (NAbs) or complement-mediated lysis (CL) titers (ANOVA, n = 36, $P = 0.104$; n = 36, $P = 0.091$ for NAbs and CL respectively; Fig 3). During the second phase of growth (day 105 – day 210), where both treatment groups were offered 60% of their mass weekly in multiple feeding to simulate *ad libitum* feeding, both dietary treatment groups decreased their mean Nabs and CL titer (ANOVA, n = 33, $P = 0.003$; n = 42, $P = 0.887$ for NAbs titers in high and low diet treatment groups respectively; n = 33, $P = 0.0008$; n = 42, $P = 0.485$ for CL titers in high diet and low diet treatment groups respectively; Fig 3) and thus at the end of the second growth phase no difference in NAbs or CL titers between the two treatment groups was exhibited (ANOVA, n = 39, $P = 0.865$; n = 39, $P = 0.957$ for NAbs and CL respectively; Fig 3).

There was a strong positive correlation between natural antibody and complement-mediated lysis titers in male *T.marcianus* (Correlation, r = 0.88, df = 61, $P < 0.0001$; Fig. 1b). The two-way ANOVA determined that overall there was no significance between diet and growth phases on the mean number of agglutination titers ($F_{1, 62} = 1.125$, $P = 0.293$) and mean lysis titers and growth phases ($F_{1, 62} = 0.757$, $P = 0.388$). At the end of the first growth phase the high diet and low diet treatment groups displayed no difference in NAbs or CL titers (ANOVA, n = 33, $P = 0.209$; n = 33, $P = 0.307$ for NAbs and CL respectively; Fig 4). During the second phase of growth neither the high diet treatment nor low diet treatment groups significantly changed their mean number of NAbs and CL titers (ANOVA, n = 31, $P = 0.140$; n = 32, $P = 0.896$ for NAbs titers in high and low diet treatment groups respectively; n = 31, $P = 0.126$; n = 32, $P = 0.734$ for CL titers in high diet and low diet treatment groups respectively; Fig 4). Thus

there was no difference in the mean number of NAbs and CL titers between the two dietary groups at the end of the second phase of growth (ANOVA, n = 30, $P = 0.976$; n = 30, $P = 0.966$ for NAbs and CL respectively; Fig 4).

There was a strong positive correlation between natural antibody and complement-mediated lysis titers in female *P.guttata* (Correlation, r = 0.97, df = 34, $P < 0.0001$; Fig 2a). The two-way ANOVA determined that overall there was no significance between diet and growth phases on mean number of agglutination titers ($F_{1, 23} = 1.197$, $P = 0.287$) and mean lysis titers and growth phases ($F_{1, 23} = 0.281$, $P = 0.602$). At the end of the first growth phase (day 175) the two dietary treatments showed no difference in mean number of NAbs or CL titers (ANOVA, n = 13, $P = 0.552$; n = 13, $P = 0.288$ for NAbs and Lysis respectively; Fig 5). During the second growth phase both treatment groups increased their mean number of NAbs and CL titers (ANOVA, n = 13, $P = 0.017$; n= 13, $P = 0.0005$ for NAbs titers in high diet and low diet treatment groups respectively; n =13, $P = 0.006$; n = 13, $P = 0.001$ for CL titers in high diet and low diet treatment groups respectively; Fig 5) and at the end of the second growth phase (day 350) no difference in mean titers between dietary treatment groups was exhibited (ANOVA, n = 13, $P = 0.997$; n = 13, $P = 0.704$ for NAbs and CL titers respectively; Fig 5).

There was a strong positive correlation between natural antibody and complement-mediated lysis titers in male *P.guttata* (Correlation, r = 0.95, df = 35, $P < 0.0001$; Fig 2b). The two-way ANOVA indicated that overall there was no significance between diet and growth phases on mean number of agglutination titers ($F_{1, 48} = 0.044$, $P = 0.835$) and mean lysis titers and growth phases ($F_{1, 48} = 0.050$, $P = 0.824$). At the end of the first growth phase the mean number of NAbs and CL titers was indistinguishable

between the high diet and low diet treatment groups (ANOVA, n = 24, $P = 0.995$; n = 24, $P = 0.979$ for NAbs and CL titers respectively; Fig 6). During the second growth phase both treatment groups displayed an increase in their mean number of NAbs and CL titers (ANOVA, n = 26, $P = 0.0002$; n= 25, $P = 0.0007$ for NAbs titers in high diet and low diet treatment groups respectively; n = 26, $P = 0.0003$; n = 25, $P = 0.0008$ for CL titers in high diet and low diet treatment groups respectively; Fig 6) thus at the end of the second growth phase there remained no difference in the mean number of NAbs or CL titers between the two dietary treatments (ANOVA, n = 27, $P = 0.999$; n = 27, $P = 0.999$; Fig 6).

Bactericidal Competence of Plasma

At the end of the first growth phase, female *T.marcianus* in the high diet treatment group displayed higher bactericidal competence (higher percent killing) than the low diet treatment group (Mann-Whitney U test, n = 34, U = 228, $P = 0.004$; Fig 7a). During the second growth phase the high diet treatment group showed no change in bactericidal competence while the low diet treatment group increased their mean percent killed (Wilcoxon test, n = 32, W = 66, $P = 0.939$; n = 36, W = 16, $P = 0.001$ for the high diet and low diet treatment groups respectively Fig 7a). Due to the increase in the low diet treatments competence, at the end of the second growth phase there was no difference in the percent killing between the two dietary treatment groups (Mann-Whitney U test, n = 38, U = 136, $P = 0.241$; Fig 7a).

The bactericidal competence at the end of the first growth phase was indistinguishable between the male *T.marcianus* dietary treatment groups (Mann-Whitney U test, n = 27, U = 113, $P = 0.296$; Fig 7b). During the second growth phase

neither dietary group displayed a significant change in bactericidal competence (Wilcoxon, n = 30, W = 23, $P = 0.124$; n = 28, W = 17, $P = 0.050$ for the high diet and low diet treatment groups respectively; Fig 7b). At the end of the second growth phase both dietary treatment groups remained indistinguishable in their bactericidal competence (Mann-Whitney U test, n = 30, U = 149, $P = 0.123$; Fig 7b).

At the end of the first growth period female *P.guttata* dietary groupings displayed no difference in their bactericidal competence (Mann-Whitney U test, n = 11, U = 9, $P = 0.329$; Fig 8a). During the second growth period the high diet treatment displayed no change in mean percent killing, while the low diet treatment group showed increases in percent killing, though not by a significant margin in percent killing (Wilcoxon test, n = 10, W = 13, $P = 0.188$; n = 10, W = 0, $P = 0.063$ for the high diet and low diet treatment groups respectively; Fig 8a). The increase in the low diet treatment groups bactericidal competence allowed it to show a greater mean bactericidal competence than the high diet treatment group at the end of the second growth phase (Mann-Whitney U test, n = 11, U = 0, $P = 0.004$; Fig 8a).

At the end of the first growth period male *P.guttata* dietary treatment groupings were indistinguishable (Mann-Whitney U test, n = 17, U = 28.5, $P = 0.549$; Fig 8b). During the second growth phase neither the high diet treatment group nor low diet treatment group displayed a change in bactericidal competence (Wilcoxon, n = 20, W = 18, $P = 0.208$; n = 14, W = 2, $P = 0.094$ for the high diet and low diet treatment groups respectively; Fig 8b). Thus, at the end of the second growth phase the low diet treatment group displayed a higher bactericidal competence than the high diet treatment group (Mann-Whitney U test, n = 24, U = 27, $P = 0.012$; Fig 8b).

Lymphocyte Abundance

Overall there was significance between diet and growth phases on mean number of lymphocytes for female *T.marcianus* (ANOVA, $F_{1, 71} = 4.884$, $P = 0.031$). At the end of the first growth phase the high diet treatment group displayed lower lymphocyte abundance on average than the low diet treatment group (ANOVA, n = 36, $P = 0.008$; Fig 9a). During the second growth phase the high diet treatment group increased their mean lymphocyte abundance though not by a significant margin and thus no significant change in lymphocyte abundance occurred during the second growth phase for either treatment group (ANOVA, n = 33, $P = 0.485$, n = 41, $P = 0.324$ for high diet and low diet treatment groups respectively; Fig 9a). At the end of the second growth phase no significant difference was seen between treatment groups (ANOVA, n = 36, $P = 0.997$; Fig 9a).

There was no difference between diet and growth phase on mean number of lymphocytes for male *T.marcianus* (ANOVA, $F_{1, 63} = 0.240$, $P = 0.626$). Male *T.marcianus* exhibited no difference in mean lymphocyte abundance between the two dietary treatment groups at the end of the first growth phase (ANOVA, n = 32, $P = 0.995$; Fig 9b). Neither treatment group displayed a significant change in lymphocyte abundance during the second growth phase (ANOVA, n = 0.34, $P = 0.908$, n = 30, $P = 0.566$ for high diet and low diet treatment groups respectively; Fig 9b) and thus at the end of the second growth phase abundance between the treatment groups remained indistinguishable (ANOVA, n = 32, $P = 0.784$; Fig 9b).

There was no difference between diet and growth phase on mean number of lymphocytes for female *P.guttata* (ANOVA, $F_{1, 22} = 0.007$, $P = 0.935$). At the end of the first growth phase, the two dietary treatment groups for female *P.guttata* displayed no

significant difference in lymphocyte abundances (ANOVA, n = 11, $P = 0.887$; Fig 10a). During the second growth phase neither the high diet treatment group nor the low diet treatment group changed its mean abundance (ANOVA, n = 12, $P = 0.506$, n = 11, $P = 0.614$ for high diet and low diet treatment groups respectively; Fig 10a) thus at the end of the second growth phase lymphocyte abundance remained indistinguishable between the treatment groups (ANOVA, n = 12, $P = 0.919$; Fig 10a).

There was a significant difference between diet and growth phase on mean number of lymphocytes for male *P.guttata* (ANOVA, $F_{1, 45} = 10.743$, $P = 0.002$). At the end of the first growth phase, the two dietary treatment groups for male *P.guttata* displayed no significant difference in lymphocyte abundances (ANOVA, n = 23, $P = 0.070$; Fig 10b). During the second growth phase the high diet treatment group increased its mean abundance (ANOVA, n = 22, $P = 0.004$, n = 0.24, $P = 0.789$ for high diet and low diet treatment groups respectively; Fig 10b) however at the end of the second growth phase lymphocyte abundance remained indistinguishable between the treatment groups (ANOVA, n = 23, $P = 0.167$; Fig 10b).

Heterophil/Lymphocyte Ratio

There was no difference between diet and growth phase on mean heterophil/lymphocyte ratios for female *T.marcianus* (ANOVA, $F_{1, 71} = 0.017$, $P = 0.898$). The high diet treatment group for female *T.marcianus* displayed higher mean heterophil/lymphocyte (H/L) ratios than the low diet treatment group at the end of the first growth phase (ANOVA, n = 36, $P = 0.009$; Fig 11a). Both treatment groups displayed an increase their H/L ratio during the second growth phase (ANOVA, n = 33, $P = 0.078$, n = 41, $P = 0.023$ for high diet and low diet treatment groups respectively; Fig

11a) and thus at the end of the second growth phase the high diet treatment group continued to display a higher H/L ratio on average (ANOVA, n = 36, $P = 0.017$; Fig 11a).

There was no difference between diet and growth phase on mean heterophil/lymphocyte ratios for male *T.marcianus* (ANOVA, $F_{1, 63} = 2.432$, $P = 0.124$). Male *T.marcianus* exhibited no difference in mean H/L ratios between the two dietary treatment groups at the end of the first growth phase (ANOVA, n = 32, $P = 0.872$; Fig 11b). The low diet treatment group displayed a significant change in H/L ratio during the second growth phase (ANOVA, n = 34, $P = 0.783$, n = 30, $P = 0.019$ for high diet and low diet treatment groups respectively; Fig 11b) however at the end of the second growth phase H/L ratios between the treatment groups remained indistinguishable (ANOVA, n = 32, $P = 0.476$; Fig 11b).

There was a significant difference between diet and growth phase on mean heterophil/lymphocyte ratios for female *P.guttata* (ANOVA, $F_{1, 22} = 16.47$, $P = 0.0007$). Female *P.guttata* exhibited no H/L ratio differences between dietary treatment groups at the end of the first growth phase (ANOVA, n = 11, $P = 0.141$; Fig 12a). During the second growth phase the low diet treatment group decreased its average H/L ratio while the high diet treatment group displayed no change (ANOVA, n = 0.12, $P = 0.165$, n = 11, $P = 0.011$ for high diet and low diet treatment groups respectively; Fig 12a). Despite the low diet treatment groups decrease in H/L ratio at the end of the second growth period the dietary groupings were indistinguishable from one another (ANOVA, n = 12, $P = 0.012$; Fig 12a).

There was no difference between diet and growth phase on mean heterophil/lymphocyte ratios for male *P.guttata* (ANOVA, $F_{1, 45} = 0.560$, $P = 0.458$).

Male *P.guttata* exhibited no difference in mean H/L ratios between the two dietary treatment groups at the end of the first growth phase (ANOVA, n = 23, $P = 0.979$; Fig 12b). Neither treatment group displayed a significant change in H/L ratio during the second growth phase (ANOVA, n = 22, $P = 0.981$, n = 24, $P = 0.903$ for high diet and low diet treatment groups respectively; Fig 12b) and thus at the end of the second growth phase H/L ratios between the treatment groups remained indistinguishable (ANOVA, n = 23, $P = 0.910$; Fig 12b).

DISCUSSION

These results demonstrate the role that immunity, both constitutive innate and adaptive, plays with the interaction of natal nutrition and growth rates of individuals in two species of snake that differ in sexual size dimorphism. It was hypothesized that adaptive immunity, and potentially innate responses (depending on the amount of energy allocation), will trade-off with growth in low diet treatment animals for which compensation in size occurs after a period of natal stress (i.e. male checkered garter snakes). Adaptive immunity is hypothesized to trade-off more readily with growth because the ability to mount responses against pathogens at an adaptive level is more costly than an innate level of immunity (Klasing and Leshchinsky, 1999; Lee, 2006). It was also hypothesized that a decrease in adaptive immunity, and potentially innate responses, would occur in the low diet animals (regardless the species and sex) that do not catch-up to their non-stressed conspecifics. A downgrading of immunity is hypothesized to occur in animals displaying partial compensation if the level of energy allocation towards growth were greater than would be if nutritional stress were not present, i.e. when higher than normal growth rates occur.

NATURAL ANTIBODIES AND COMPLEMENT-MEDIATED LYSIS

Natural antibodies (NAbs) and complement-mediated lysis (CL) are components of constitutive innate humoral immunity, which provides the first-line of protection against invading microbes (Matson et al., 2005). NAbs are non-specific defenses which recognize a broad array of pathogens and promote their opsonization (marking for destruction) and phagocytosis (Ochsenbein and Zinkernagel, 2000; Matson et al., 2005). Additionally, NAbs can activate the complement enzyme cascade, which results in the formation of killing complexes on the surface of invading pathogens, leading to their lysis (Ochsenbein and Zinkernagel, 2000; Matson et al., 2005). The CL measured in this study is a result of the interaction of both NAbs and complement proteins (Lancaster et al., 2008). Antibodies from induced immune responses could also be responsible for resulting agglutination and lysis. However, study animals maintained healthy body conditions throughout the experiment and were not subjected to pathogens to stimulate an induced immune response, and thus I concluded that only natural antibodies were being quantified. Thus the assay specific hypotheses for NAbs and CL was that a decrease in innate humoral immunological competency would not occur unless there was a large shift in the allocation of energy into growth (as seen with compensatory growth) to trade-off with both adaptive and innate immune components.

Female (Thamnophis marcianus)

Average agglutination (NAbs) and lysis (CL) titers were not different between the high and low treatment groups after the first growth phase ($P = .104$, $P = .091$ for agglutination and lysis titers respectively) and remained indistinguishable after the second growth phase ($P = .865$, $P = .957$ for agglutination and lysis titers respectively).

Thus I found that female *T.marcianus* that even with a partial compensatory response (faster growth rates in the low diet group with an increase in food availability) there was no downgrading of the constitutive innate immune response based on the natural antibodies and complement-mediated lysis parameters. Thus the results can perhaps better fit one of two hypotheses: That compensatory growth did not occur and excess energy, from increased resources, was not allocated into growth and thus no change in immunological parameters would be expected. Or that even though compensatory growth did not occur, resources were allocated to growth to partially compensate for size deficiency, but that allocation did not cause a downgrading of immunological function for the constitutive innate humoral components of NAbs and CL.

Male (Thamnophis marcianus)

Average agglutination (NAbs) and lysis (CL) titers were not different between the high and low treatment groups after the first growth phase ($P = .209$, $P = .307$ for agglutination and lysis titers respectively) and remained indistinguishable after the second growth phase ($P = .976$, $P = .966$ for agglutination and lysis titers respectively). These results suggest that although more energy was allocated into growth, following a period of growth depression (for compensation to occur) that the shift in energy did not cause a downgrading of the constitutive innate humoral components of NAbs and CL.

Female (Pantherophis guttata)

Average agglutination (NAbs) and lysis (CL) titers were not different between the high and low treatment groups after the first growth phase ($P = .552$, $P = .288$ for agglutination and lysis titers respectively). During the second growth phase both

treatment groups increased their average number of titers ($P = .017$, $P = .0005$ for high and low diets agglutination titers respectively; $P = .006$, $P = .001$ for high and low diet lysis titers respectively) and remained indistinguishable after the second growth phase ($P = .997$, $P = .704$ for agglutination and lysis titers respectively). Thus I found that female *P.guttata* showed similarity to female checkered garter snakes by not showing a downgrading of constitutive innate humoral immunity, through natural antibodies and complement mediated lysis, following a period of depressed growth.

Male (Pantherophis guttata)

Average agglutination (NAbs) and lysis (CL) titers were not different between the high and low treatment groups after the first growth phase ($P = .995$, $P = .979$ for agglutination and lysis titers respectively) similar to male *T.marcianus*. However, during the second growth phase both treatment groups increased their average number of titers ($P = .0002$, $P = .0007$ for high and low diets agglutination titers respectively; $P = .0002$, $P = .0008$ for high and low diet lysis titers respectively) and remained indistinguishable after the second growth phase ($P = .999$, $P = .999$ for agglutination and lysis titers respectively). These results indicate that increased growth rates in low diet animals after a phase of depressed growth does not cause a downgrading in constitutive innate humoral immune responses, specifically within natural antibodies and complement-mediated lysis immune parameters. Thus the results can potentially better-fit one of two hypotheses: That compensatory growth did not occur and excess energy, through increased resources, was not allocated into growth and thus no change in immunological parameters would be expected. Or that even though compensatory growth did not occur, resources were allocated to growth to partially compensate for size deficiency, but that allocation did not

cause a downgrading of immunological function for the constitutive innate humoral components of NAbs and CL.

Summary

Although immune function is expensive to maintain and has been found to trade-off with other resource-demanding physiological processes, I found little evidence to support the hypothesis that natural antibodies and complement-mediated lysis innate immune constituents trade-off with growth following periods of nutritional stress for either species or either sex. The lack of association between growth allocation and natural antibodies might not be surprising as their production is thought to be fairly independent of internal and external stimuli (Ochsenbein and Zinkernagel, 2000). Additionally, recent studies with poultry suggest that the developmental costs of both NAbs and complement proteins are relatively low (Hau et al., 2010) and therefore, condition-dependence may only become apparent in cases of high nutritional stress causing very poor body condition (Palacios et al., 2012). Therefore, a downgrading of the NAbs and CL components of constitutive innate humoral immunity may not be affected unless severe levels of malnutrition are reached, suggesting that a depressed growth rate due to natal nutritional stress as displayed in this study may not have been a strong enough response to stimulate an immune response. Alternatively, it may also indicate that NAbs and CL innate immune parameters are maintained at the expense of other behavioral or physiological mechanisms.

BACTERICIDAL COMPETENCE

Bactericidal competence of plasma is a measure of the integrated bacterial killing capacity of plasma proteins, including natural antibodies and complement, as well as lysozyme and constitutively produced acute phase proteins and constitutes a component of innate humoral immunity (Matson et al., 2006). A higher bactericidal competence correlates to a higher in vitro killing percentage of a nonpathogenic strain of *E. coli*, killing invasive bacteria is a fundamental immune function (Matson et al., 2006). Due to the innate immune properties of this assay, the assay specific hypothesis for bactericidal competence was that a decrease in innate humoral immunological competency (i.e. percent killing) would not occur unless there was a large shift in the allocation of energy into growth (as seen with compensatory growth) to trade-off with innate immune components such as bacterial killing capacity. This has been seen in several bird species such as the blue-crowned motmot (*Momotus momota*), the blue gray tanager (*Turdus grayi*), crimson-backed tanagers (*Ramphocelus dimidiatus*) and clay-colored robins (*Columbina talpacoti*) (Matson et al., 2006).

Female (Thamnophis marcianus)

Following the first growth phase, there was a difference in the percent killing between the high and low diet treatment groups for female *T.marcianus* ($P = .004$). During the second growth phase the low diet treatment group increased it's bactericidal competence ($P = .001$) and thus at the end of the second growth phase there was no difference between the treatment groups ($P = .241$). Following a period of nutritional stress, female *T.marcianus* displayed no downgrading of innate immunity through bactericidal competence suggesting that allocating resources into increased growth rates

does not have an effect on the constitutive innate humoral immune response. The results instead better fit one of two hypotheses: either compensatory growth did not occur and the energy acquired from increased resources was not allocated into growth and thus no change in immunological parameters would be expected. Or that even though compensatory growth did not occur, resources were still allocated towards growth and to partially compensate for size deficiency, but that allocation did not cause a downgrading of immunological function for the constitutive innate component of bactericidal competence. However, if a downgrading of immunological function is not occurring due to growth, it could be suggested that the difference in killing capacity between the treatment groups at the end of the first growth phase (low diet treatment group displayed lower average killing capacity, Table 5) indicates a sensitivity of this immune component to nutritional stress.

Male (Thamnophis marcianus)

Mean percent killing was not different between the high and low treatment groups after the first growth phase ($P = .296$) and remained indistinguishable after the second growth phase ($P = .123$) for male *T.marcianus*. These results indicate that although resources were allocated towards growth, following a phase of nutritional stress and thus depressed growth, for compensation to occur that the shift in energy did not cause a downgrading of the constitutive innate humoral components of bactericidal competence (Table 6).

Female (Pantherophis guttata)

Mean percent killing was not different between the dietary treatment groups after the first growth phase ($P = .329$). Although neither treatment group increased nor decreased their bactericidal competence by a significant margin ($P = .188$; $P = .063$ for high and low diet treatment groups respectively) there was a difference in mean percent killing at the end of the second growth phase ($P = .004$). The difference in killing capacity however was not as anticipated; the low diet treatment group increased their competence to be on average higher than the high diet treatment group. Similar to female *T.marcianus,* this suggests that a down-regulation of innate parameters of bactericidal competence does not occur in response to depressed growth rates as seen in periods of nutritional stress.

Male (Pantherophis guttata)

Mean percent killing was not different between the dietary treatment groups for male *P. guttata* after the first growth phase ($P = .549$). Although neither treatment group increased nor decreased their bactericidal competence by a significant margin ($P = .208$; $P = .093$ for high and low diet treatment groups respectively) there was a difference in mean percent killing at the end of the second growth phase ($P = .012$). The difference in killing capacity however was not as anticipated; the low diet treatment group increased their competence to be on average higher than the high diet treatment group. This suggests that a down-regulation of the innate parameter of bactericidal competence does not occur in response to depressed growth rates as seen in periods of nutritional stress. However, if a downgrading of immunological function is not occurring due to growth, it could be suggested that the difference in killing capacity between the treatment groups at

the end of the first growth phase is indicative of the innate immune component of bactericidal competence being sensitive to nutritional stress such that it is slightly suppressed when nutrient availability is limited.

Summary

Overall I found little evidence to support the hypothesis that the innate immune constituent of bactericidal competence would trade-off with growth following periods of nutritional stress. Killing ability has been shown to vary significantly across species (Matson et al., 2006) and is predicted to relate to where a particular species falls on the slow-fast continuum of life-history variation and also how it correlates with other variables such as rates of reproduction and development (Ricklefs and Wikelski, 2002). Stress was also a factor which appeared to reduce the killing capacity of the plasma components; studies by Matson et al. (2006) showed that one hour of acute stress reduced killing ability by up to 40% and thus I would assume that chronic levels of stress, such as that brought on by nutritional deficiencies may be the cause for the effects seen on bacterial killing percentages in the male and female *P.guttata*.

LYMPHOCYTE ABUNDANCE

Levels of circulating lymphocytes can be utilized as an assessment of acquired immunity. Lymphocytes are considered the major players of the acquired immune system and thus their abundance may be used as a measure of investment in induced acquired immunity (Lee, 2006). Although lymphocyte proliferation occurs when an organism is subjected to a pathogen, all study animals maintained healthy body conditions throughout the duration of the study and thus proliferation due to acute

infection can be ruled out. It has been previously demonstrated that chronic stressors, such as periods of nutritional stress, elicit the same responses on immunological processes as exposure to pathogens. Thus the assay specific hypothesis was that a downgrading of adaptive immunity should occur with the allocation of excess energy into accelerating growth rates, especially if those growth rates are high enough to allow for compensation in size by the low diet treatment groups.

Female (Thamnophis marcianus)

At the end of the first growth phase there was a difference in lymphocyte counts between the two dietary treatment groups for female *T.marcianus* ($P = .007$), lymphocyte counts on average were higher for the low diet treatment group. By the end of the second growth phase there was no difference between the two dietary groupings ($P = .997$). These results suggest that allocating resources into growth following periods of growth depression does not cause a down-regulation of adaptive immune responses as displayed by mean lymphocyte abundance. Thus these results suggest that a greater investment in acquired immunity was being made to the low diet treatment group during the first growth phase, when it was expected that if energy allocation and acquired immune responses traded off that I would view the opposite trend. Although I saw no indication of poor health, it could be indicative of infection within a subset of the animals, driving up the lymphocyte number, rather than an overall up regulation of acquired immunity.

Male (Thamnophis marcianus)

At the end of the first growth phase there was no difference between the high and low dietary treatment groups for male *T.marcianus* ($P = .995$). During the second growth

phase, neither treatment group increased or decreased their lymphocyte numbers by a significant margin and thus at the end of the second growth phase the two treatment groups showed no difference in lymphocyte abundance ($P = .784$). These results suggest that a growth depression followed by an increased rate of growth to allow for overall size compensation does not cause a down-regulation of the adaptive immune component of mean lymphocyte abundance.

Female (Pantherophis guttata)

At the end of the first growth phase there was no difference between the high and low dietary treatment groups for female *P.guttata* ($P = .887$). During the second growth phase, neither treatment group increased or decreased their lymphocyte numbers by a significant margin and thus at the end of the second growth phase there was no difference between the two treatment groups ($P = .919$). These results suggest that increased growth rates (to promote a trend toward size compensation) do not cause a downgrading of the adaptive immune component of mean lymphocyte abundance. These results instead suggest that the data better fit one of two hypotheses: either compensatory growth did not occur and excess energy was not allocated into growth and thus no change in immunological parameters would be expected. Or that even though compensatory growth did not occur excess energy was still allocated to growth to partially compensate for size deficiency, but that energy allocation did not cause a downgrading of immunological function for the induced acquired immune response.

Male (Pantherophis guttata)

At the end of the first growth phase there was no difference between the high and low dietary treatment groups for male *P.guttata* ($P = .070$). During the second growth phase, neither treatment group increased or decreased their lymphocyte numbers by a significant margin and thus at the end of the second growth phase there was no difference between the two treatment groups ($P = .167$). These results suggest that allocating resources into growth following periods of growth depression does not cause a down-regulation of adaptive immune responses as displayed by mean lymphocyte abundance. Instead, the data may better fit one of two hypotheses: either compensatory growth did not occur and excess energy was not allocated into growth and thus no change in immunological parameters would be expected. Or that even though compensatory growth did not occur resources were still allocated to growth to partially compensate for size deficiency, but that allocation did not cause a downgrading of immunological function for the induced acquired immune response of lymphocyte abundance.

Summary

Overall I found little evidence to support the hypothesis that the allocation of excess energy sources into growth to cause compensation would trade-off with acquired immunity. Since lymphocyte abundance is only a rough index of acquired immunity, other components should be evaluated in order to test this prediction more thoroughly (Sparkman et al., 2009). A potentially more accurate means of determining adaptive immune function would be to subject the animals to a pathogen and measure their immune function prior to infection and their response to the infection. Any conclusions based on mean lymphocyte numbers must be interpreted cautiously, as lymphocyte

abundances can be higher in individuals battling infections and thus false positive results on up regulated acquired immune systems may occur (Sparkman et al., 2007; 2009). However, all animals within the study maintained healthy body conditions throughout the experiment and were not subject to visible infections and thus mean lymphocyte abundance was considered a reliable parameter for overall adaptive immune function.

HETEROPHIL TO LYMPHOCYTE RATIO

Levels of circulating heterophils compared to levels of circulating lymphocytes (heterophil to lymphocyte ratio) can be utilized as an index to stress (Gross and Siegel, 1983, Vleck et al., 2000). Stressors such as food or water deprivation elevate the number of heterophils and depress the number of lymphocytes (Vleck et al., 2000). The heterophil to lymphocyte ratio is also deemed a good test for stress because compared to the glucocorticoids, leukocyte numbers change much more slowly in response to stress; these changes are also less variable, longer lasting and multiple stressors tend to have an additive affect (Vleck et al., 2000).

Female (Thamnophis marcianus)

At the end of the first growth phase there was a difference between the high and low dietary treatment groups for female *T.marcianus* ($P = .009$). During the second growth phase, both treatment group increased their heterophil to lymphocyte ratios by a significant margin and thus at the end of the second growth phase there remained a difference between the two treatment groups ($P = .016$). Since both treatment groups showed marked increases in their heterophil to lymphocyte ratio, it is unlikely that the

animals were allocating excess energy into growth and sustaining a trade-off in leukocyte percentages.

Male (Thamnophis marcianus)

At the end of the first growth phase there was no difference between the high and low dietary treatment groups for male *T.marcianus* ($P = .872$). During the second growth phase, only the low diet treatment group increased their heterophil to lymphocyte ratios however, at the end of the second growth phase there remained no difference between the two treatment groups ($P = .476$). These results suggest that even though compensatory growth occurred, the allocation of excess resources into growth did not cause a trade-off with heterophil to lymphocyte ratios.

Female (Pantherophis guttata)

At the end of the first growth phase there was no difference between the high and low dietary treatment groups for female *P.guttata* ($P = .141$). During the second growth phase, the low diet treatment group decreased their heterophil to lymphocyte ratios and thus at the end of the second growth phase there was a difference between the two treatment groups ($P = .012$). These results suggest one of two things, either that partial compensatory growth and thus allocation of excess energy into growth did not occur and the downgrading of the heterophil to lymphocyte ratio is a residual decrease in numbers when the stressor of food deprivation is no longer present. Or that even though complete compensation did not occur, excess energy was still allocated to growth to partially compensate for size deficiency and that energy did not cause a downgrading of immunological function and thus the change in heterophil to lymphocyte ratio is sensitive

to nutritional stress and is activated when low levels of food are available and suppressed when nutrient availability returns.

Male (Pantherophis guttata)

At the end of the first growth phase there was no difference between the high and low dietary treatment groups for male *P.guttata* ($P = .979$). During the second growth phase, neither treatment group decreased nor increased their heterophil to lymphocyte ratios and thus at the end of the second growth phase there remained no difference between the two treatment groups ($P = .910$). These results suggest that following a period of growth depression when increased levels of resources are present, an increase in growth rates (following a trend toward compensating in size) does not cause a downgrading of the innate and stress parameter of heterophil to lymphocyte ratio. Thus these also results suggest that the results may better fit one of two hypotheses: either compensatory growth did not occur and excess energy was not allocated into growth and thus no change in immunological parameters would be expected. Or that even though compensatory growth did not occur excess energy was still allocated to growth to partially compensate for size deficiency, but that energy allocation did not cause a downgrading of immunological function for the induced acquired immune response.

Summary

Overall I found little evidence to suggest that energy allocation to growth causes an effect on heterophil to lymphocyte ratios as a parameter of stress. This suggests that either allocation of energy to increasing growth rates does not cause a downgrading of the heterophil to lymphocyte ratio, but instead perhaps occurs at a different level of

behavioral physiological processes. High correlations between heterophil to lymphocyte ratio and level of corticosterone have been found when working with well socialized groups that had a stable social hierarchy (Gross and Siegel, 1983) and thus in populations that deviate from that level of organization utilizing more than one test for stress is beneficial.

Conclusions

I found little evidence to support the prediction that adaptive immune parameters, which are thought to be more costly to develop and maintain than innate parameters, are likely to trade-off with growth when compensation in growth due to excess energy allocation occurs after periods of nutritional stress. I also found little evidence to support predictions of down-regulation in innate immune parameters when an allocation of excess energy is put into growth. I also found that whether full or partial (a trend towards compensating by increasing growth rates) compensation occurred, there was no visible difference in results; no matter the level of post stress growth, immune function showed no down-regulation. These results suggest that either snakes are robust animals that can maintain health at low levels of food intake, or that immune function parameters are not down regulated when excess energy is allocated to compensation (either partial or complete) in growth. Nutritional stress to depress growth rates may not be strong enough stimulus to promote a downgrading of immune function, animals may need to face a severe phase of malnutrition to decrease their overall health before any immune downgrading occurs. Therefore, it is proposed that perhaps a trade-off with growth is occurring in other physiological processes or within the animal's behavioral patterns when growth rates diverge from normal to compensate for early deficiencies. These

results also suggest that maintenance of immune function, both innate and adaptive, are evolutionarily adapted to be maintained during periods of stress.

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TABLES & FIGURES

Table 5. Immune components between high and low diet female *Thamnophis marcianus* for two growth phases.

T.marcianus (female)	Growth Phase 1 (day 105)						Growth Phase 2 (Day 210)					
	High Diet			Low Diet			High Diet			Low Diet		
Immune component (units)	Mean ± SE	min-max	n	Mean ± SE	min-max	n	Mean ± SE	min-max	n	Mean ± SE	min-max	n
Lymphocytes (per 10,000 RBCs)	74.19 ± 1.69	85-64	16	81.50 ± 1.52	94-65	20	77.50 ± 1.93	86-57	16	77.95 ± 1.10	91-69	20
Heterophils (per 10,000 RBCs)	3.50 ± .42	7-1	16	2.05 ± .28	4-0	20	5.56 ± .63	10-1	16	3.30 ± .29	6-1	20
Natural Antibody (agglut. Titer)	5.97 ± .18	4.5-7.25	17	5.30 ± .21	3.5-7.0	19	4.96 ± .23	3.5-6.25	17	5.07 ± .19	4.0-6.75	22
Complement (lysis titer)	5.69 ± .19	4.25-7.25	17	5.05 ± .16	3.5-6.25	19	4.62 ± .22	3.0-6.25	17	4.66 ± .18	3.5-6.5	22
Bactericidal competence (% killed)	97.08 ± .89	86.4-99.7	16	86.72 ± 3.02	64.1-99.5	18	90.98 ± 4.46	26.1-100	17	96.40 ± 1.78	82.9-100	22

Table 6. Immune components between high and low diet male *Thamnophis marcianus* for two growth phases.

T.marcianus (male)	Growth Phase 1 (day 105)						Growth Phase 2 (Day 210)					
	High Diet			Low Diet			High Diet			Low Diet		
Immune component (units)	Mean ± SE	min-max	n	Mean ± SE	min-max	n	Mean ± SE	min-max	n	Mean ± SE	min-max	n
Lymphocytes (per 10,000 RBCs)	80.65 ± 1.11	90-74	17	80.13 ± 1.71	90-66	15	79.29 ± 1.46	89-63	17	77.33 ± 1.62	83-61	15
Heterophils (per 10,000 RBCs)	1.65 ± .19	3-1	17	1.67 ± .43	6-0	15	2.12 ± .32	4-1	17	2.73 ± .34	6-1	15
Natural Antibody (agglut. Titer)	5.74 ± .18	4.0-7.0	17	5.19 ± .23	3.25-7.0	15	4.91 ± .27	1.75-6.0	16	5.00 ± .14	4.0-6.0	15
Complement (lysis titer)	5.38 ± .20	4.0-6.75	17	4.87 ± .25	2.5-7.0	15	4.55 ± .22	2.0-5.75	16	4.58 ± .15	4.0-6.0	15
Bactericidal competence (% killed)	95.43 ± 1.85	74.7-99.7	14	94.48 ± 1.69	76.6-100	14	98.63 ± .75	88.9-100	15	98.18 ± .46	94.6-100	15

Table 7. Immune components between high and low diet female *Pantherophis guttata* for two growth phases.

P.guttata (female)	Growth Phase 1 (day 175)						Growth Phase 2 (Day 350)					
	High Diet			Low Diet			High Diet			Low Diet		
Immune component (units)	Mean ± SE	min-max	n	Mean ± SE	min-max	n	Mean ± SE	min-max	n	Mean ± SE	min-max	n
Lymphocytes (per 10,000 RBCs)	64.83 ± 3.94	51-80	6	68.40 ± 3.61	56-76	5	71.50 ± 3.08	57-78	6	74.50 ± 2.91	67-84	6
Heterophils (per 10,000 RBCs)	6.67 ± .71	5-8	6	11.40 ± .51	10-13	5	11.67 ± 1.73	8-18	6	5.67 ± .61	3-7	6
Natural Antibody (agglut. Titer)	4.25 ± .47	2.75-5.5	6	3.46 ± 44	2.0-5.25	6	6.21 ± .28	5.5-7.0	6	6.33 ± .46	5.0-7.5	6
Complement (lysis titer)	4.00 ± .45	2.5-5.5	6	3.08 ± .37	2.0-4.5	6	5.88 ± .26	5.0-6.5	6	5.33 ± .31	4.5-6.5	6
Bactericidal competence (% killed)	33.70 ± 14.07	0-72.24	5	53.64 ± 9.00	26.3-89.63	6	7.97 ± 12.97	0-34.04	6	89.40 ± 5.27	68.98-98.43	5

Table 8. Immune components between high and low diet male *Pantherophis guttata* for two growth phases.

P.guttata (male)	Growth Phase 1 (day 175)						Growth Phase 2 (Day 350)					
	High Diet			Low Diet			High Diet			Low Diet		
Immune component (units)	Mean ± SE	min-max	n	Mean ± SE	min-max	n	Mean ± SE	min-max	n	Mean ± SE	min-max	n
Lymphocytes (per 10,000 RBCs)	62.36 ± 1.46	53-69	11	69.08 ± 2.07	57-76	12	72.27 ± 1.64	63-80	11	66.00 ± 2.15	51-78	12
Heterophils (per 10,000 RBCs)	7.73 ± 1.08	3-14	11	7.58 ± 1.06	3-16	12	8.36 ± 1.66	1-21	11	9.08 ± 1.77	3-25	12
Natural Antibody (agglut. Titer)	3.89 ± .45	2.0-7.0	11	4.00 ± .24	2.5-5.0	12	5.89 ± .21	5.0-7.5	14	5.88 ± .35	4.0-7.75	12
Complement (lysis titer)	3.94 ± .36	NR-6.25	11	3.75 ± .21	2.5-4.5	12	5.34 ± .18	4.5-6.5	14	5.38 ± .31	4.0-7.75	12
Bactericidal competence (% killed)	35.45 ± 12.37	0-87.02	10	47.46 ± 12.6	0-87.04	7	29.21 ± 9.93	0-86.5	14	73.0 ± 9.69	0-99.21	10

FIGURE LEGENDS

Figure 5. Simple correlation between natural antibody and complement-mediated lysis titers in (a) female *Thamnophis marcianus* (n = 74) and (b) male *Thamnophis marcianus* (n = 62).

Figure 6. Simple correlation between natural antibody and complement-mediated lysis titers in (a) female *Pantherophis guttata* (n = 23) and (b) male *Pantherophis guttata* (n = 48).

Figure 7. Comparison of (a) natural antibody (agglutination) and (b) complement-mediated lysis titers for high and low diet treatment groups of female *Thamnophis marcianus* in the first growth phase (day 105, n = 38) and the second growth phase (day 210, n = 38). Box plots depict the median (bold horizontal line), interquartile range (height of box), range of observed values falling within a 3/2 spread of the interquartile range (whiskers), and the outliers (open circles).

Figure 8. Comparison of (a) natural antibody (agglutination) and (b) complement-mediated lysis titers for high and low diet treatment groups of male *Thamnophis marcianus* in the first growth phase (day 105, n = 31) and the second growth phase (day 210, n = 31). Box plots depict the median (bold horizontal line), interquartile range (height of box), range of observed values falling within a 3/2 spread of the interquartile range (whiskers), and the outliers (open circles).

Figure 9. Comparison of (a) natural antibody (agglutination) and (b) complement-mediated lysis titers for high and low diet treatment groups of female *Pantherophis guttata* in the first growth phase (day 175, n = 16) and the second growth phase (day 350, n = 16). Box plots depict the median (bold horizontal line), interquartile range (height of box), range of observed values falling within a 3/2 spread of the interquartile range (whiskers), and the outliers (open circles).

Figure 10. Comparison of (a) natural antibody (agglutination) and (b) complement-mediated lysis titers for high and low diet treatment groups of male *Pantherophis guttata* in the first growth phase (day 175, n = 28) and the second growth phase (day 350, n = 28). Box plots depict the median (bold horizontal line), interquartile range (height of box), range of observed values falling within a 3/2 spread of the interquartile range (whiskers), and the outliers (open circles).

Figure 11. Comparison of bactericidal competence (percent bacteria killed) for high and low diet treatment groups of (a) female *Thamnophis marcianus* and (b) male *Thamnophis marcianus* in the first growth phase (day 105, n = 34 and n = 27 for females and males respectively) and the second growth phase (day 210, n = 38 and n = 30 for females and males respectively). Bar plot represents means and standard error. Statistics provided in text.

Figure 12. Comparison of bactericidal competence (percent bacteria killed) for high and low diet treatment groups of (a) female *Pantherophis guttata* and (b) male *Pantherophis guttata* in the first growth phase (day 175, n = 11 and n = 17 for females and males respectively) and the second growth phase (day 350, n = 11 and n = 24 for females and males respectively). Bar plot represents means and standard error. Statistics provided in text.

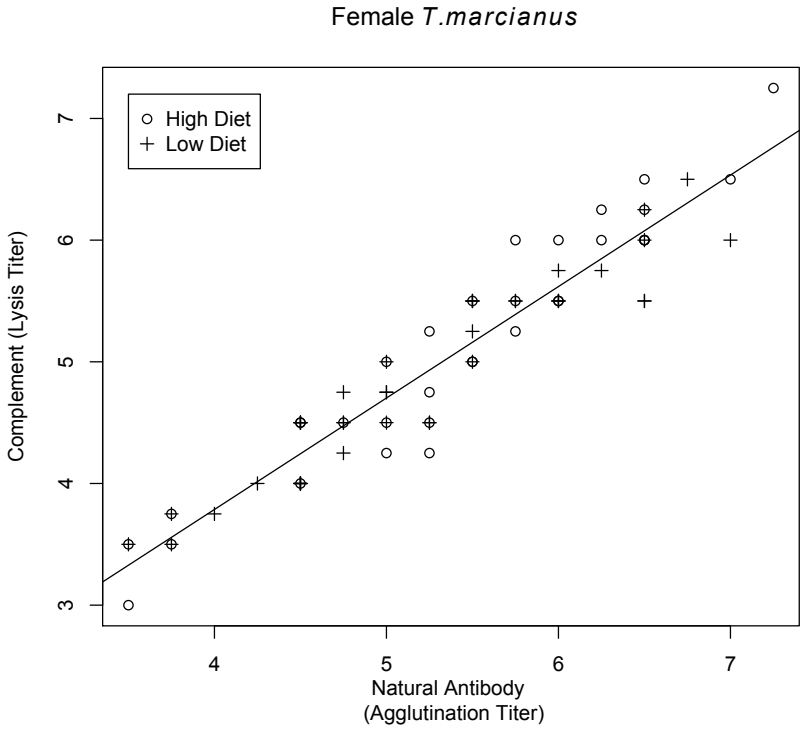
Figure 13. Comparison of lymphocyte abundances for high and low diet treatment groups of (a) female *Thamnophis marcianus* and (b) male *Thamnophis marcianus* in the first growth phase (day 105, n = 36 and n = 32 for females and males respectively) and the second growth phase (day 210, n = 36 and n = 32 for females and males respectively). Box plots depict the median (bold horizontal line), interquartile range (height of box), range of observed values falling within a 3/2 spread of the interquartile range (whiskers), and the outliers (open circles).

Figure 14. Comparison of lymphocyte abundances for high and low diet treatment groups of (a) female *Pantherophis guttata* and (b) male *Pantherophis guttata* in the first growth phase (day 175, n = 11 and n = 23 for females and males respectively) and the second growth phase (day 350, n = 12 and n = 23 for females and males respectively). Box plots depict the median (bold horizontal line), interquartile range (height of box), range of observed values falling within a 3/2 spread of the interquartile range (whiskers), and the outliers (open circles).

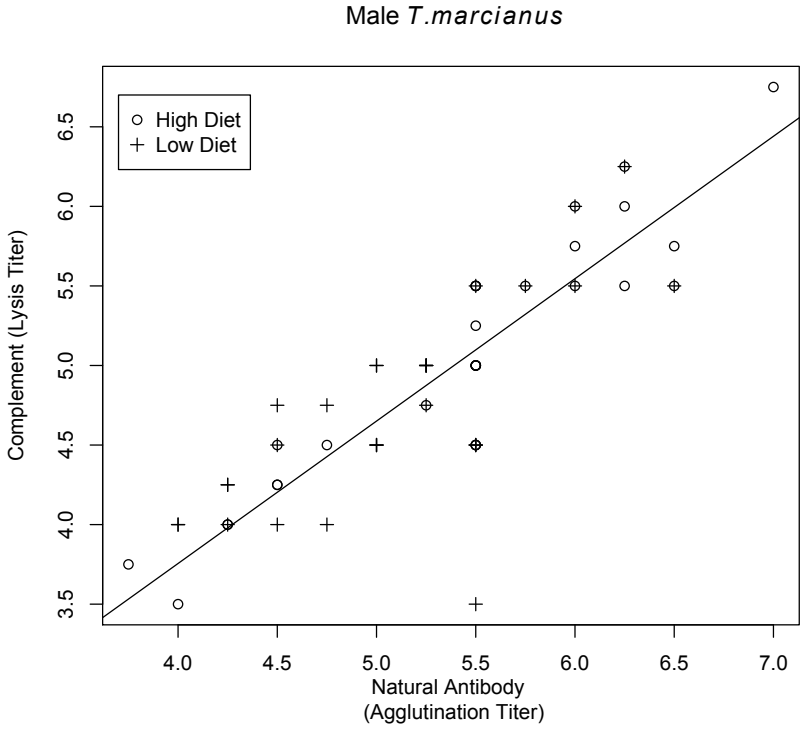
Figure 15. Comparisons of heterophil to lymphocyte (H/L) ratios for high and low diet treatment groups of (a) female *Thamnophis marcianus* and (b) male *Thamnophis marcianus* in the first growth phase (day 105, n = 36 and n = 32 for females and males respectively) and the second growth phase (day 210, n = 36 and n = 32 for females and males respectively). H/L ratios plotted as the square root transformation. Box plots depict the median (bold horizontal line), interquartile range (height of box), range of observed values falling within a 3/2 spread of the interquartile range (whiskers), and the outliers (open circles).

Figure 16. Comparison of heterophil to lymphocyte (H/L) ratios for high and low diet treatment groups of (a) female *Pantherophis guttata* and (b) male *Pantherophis guttata* in the first growth phase (day 175, n = 11 and n = 23 for females and males respectively) and the second growth phase (day 350, n = 12 and n = 23 for females and males respectively). H/L ratios plotted as the square root transformation. Box plots depict the median (bold horizontal line), interquartile range (height of box), range of observed values falling within a 3/2 spread of the interquartile range (whiskers), and the outliers (open circles).

Figure 5

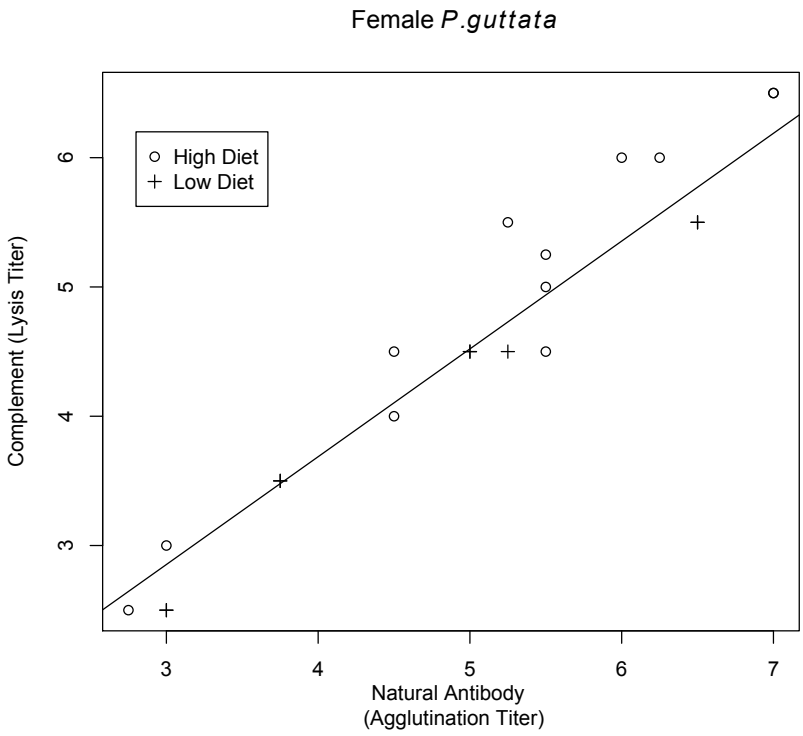

Female *T.marcianus*
High Diet
Low Diet
Complement (Lysis Titer)
Natural Antibody
(Agglutination Titer)
3
4
5
6
7

(a)

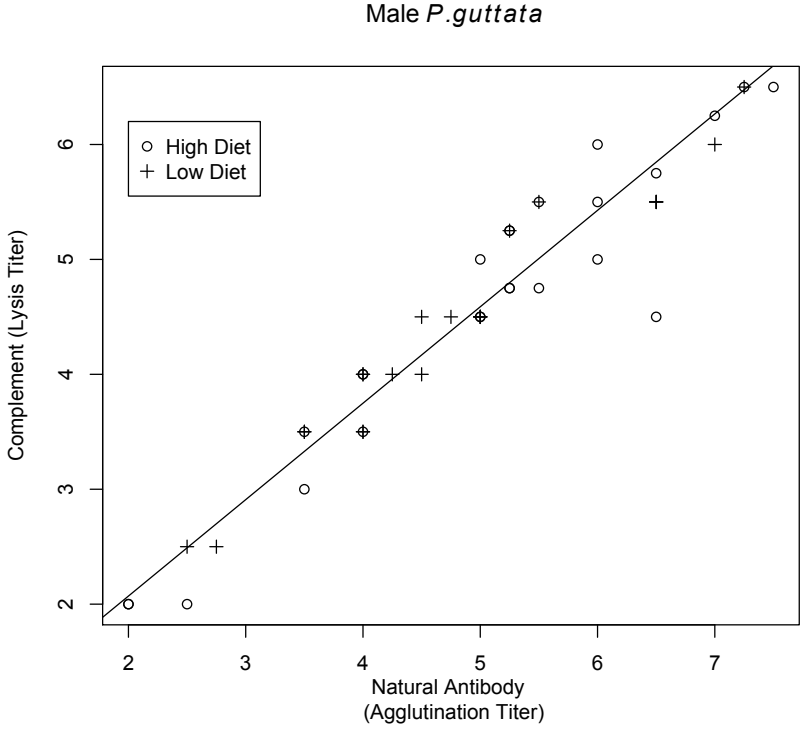

Male *T.marcianus*
High Diet
Low Diet
Complement (Lysis Titer)
Natural Antibody
(Agglutination Titer)
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0

(b)

Figure 6

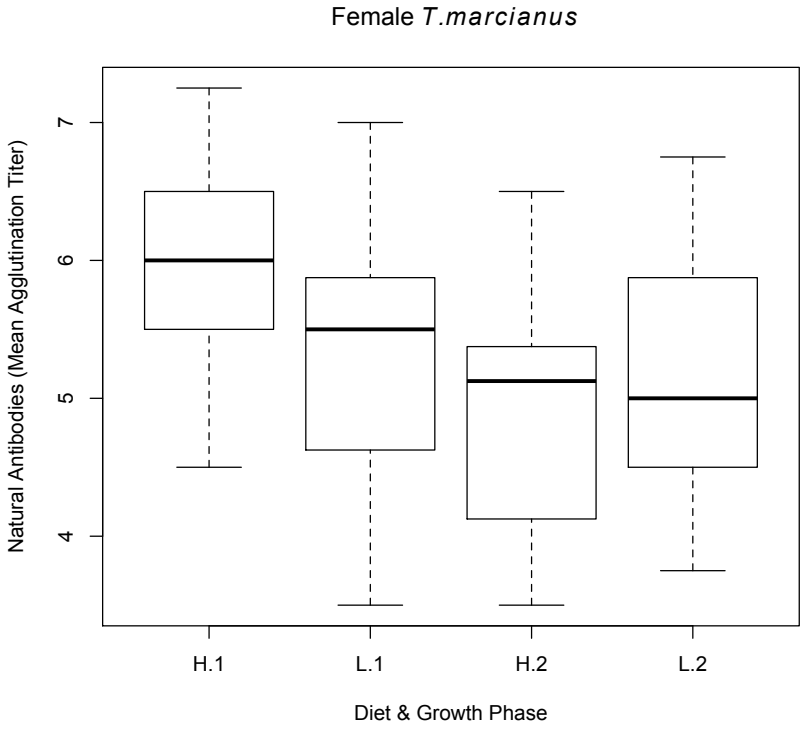

Female *P.guttata*
High Diet
Low Diet
Complement (Lysis Titer)
Natural Antibody
(Agglutination Titer)
3
4
5
6
7

(a)

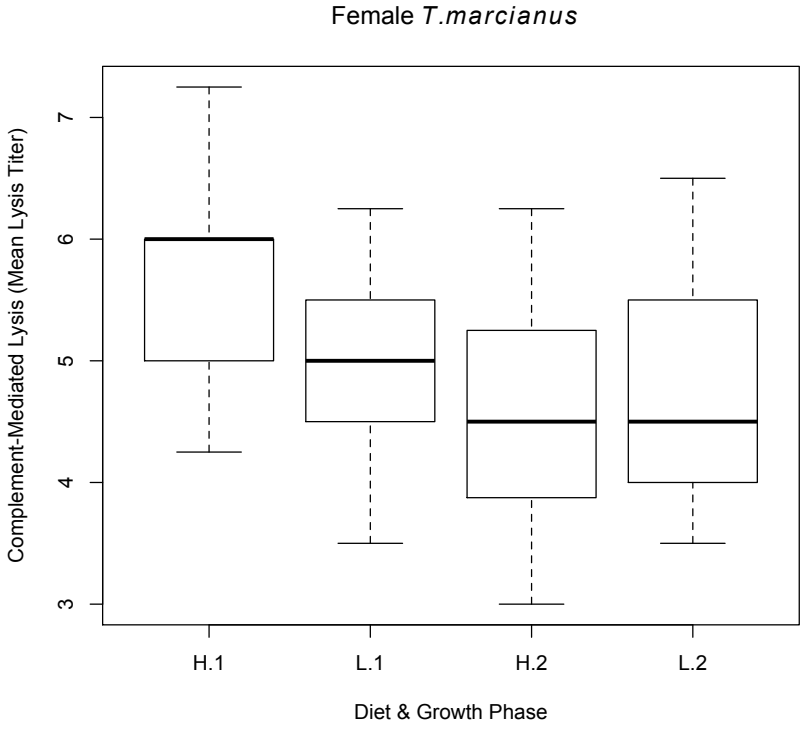

Male *P.guttata*
High Diet
Low Diet
Complement (Lysis Titer)
Natural Antibody
(Agglutination Titer)
2
3
4
5
6
7

(b)

Figure 7

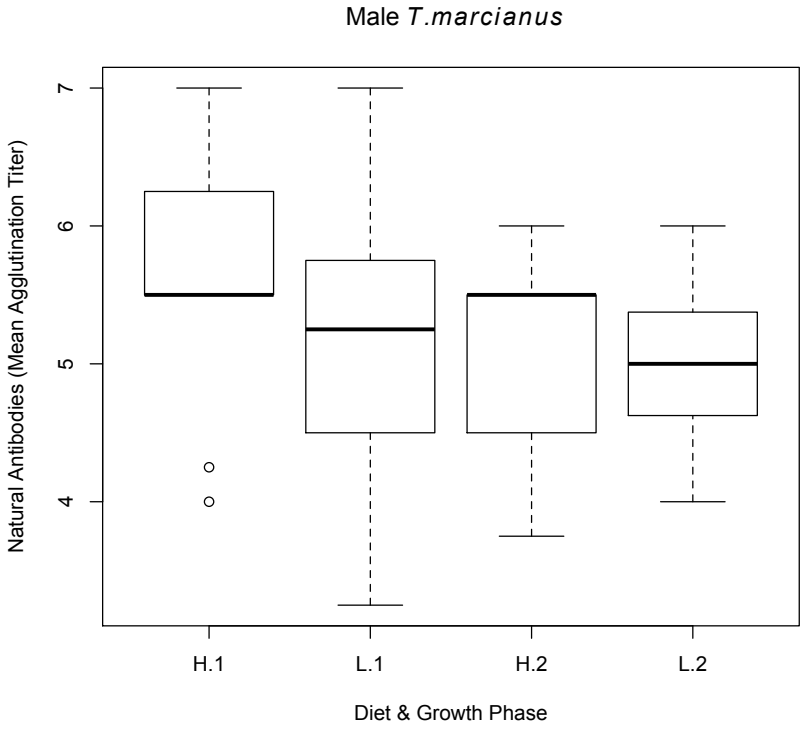

Female *T.marcianus*
Natural Antibodies (Mean Agglutination Titer)
4
5
6
7
H.1
L.1
H.2
L.2
Diet & Growth Phase

(a)

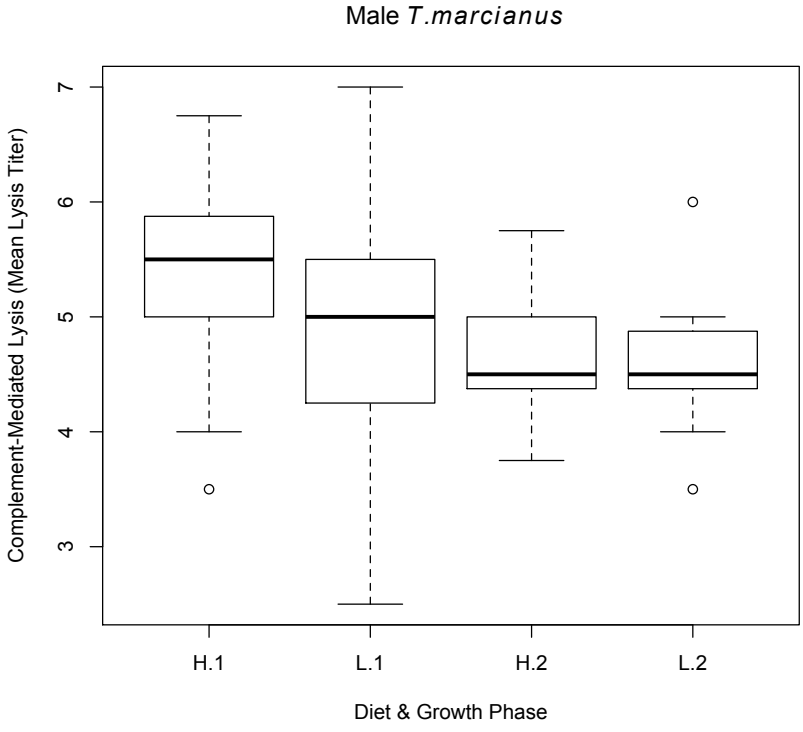

Female *T.marcianus*
Complement-Mediated Lysis (Mean Lysis Titer)
3
4
5
6
7
H.1
L.1
H.2
L.2
Diet & Growth Phase

(b)

Figure 8

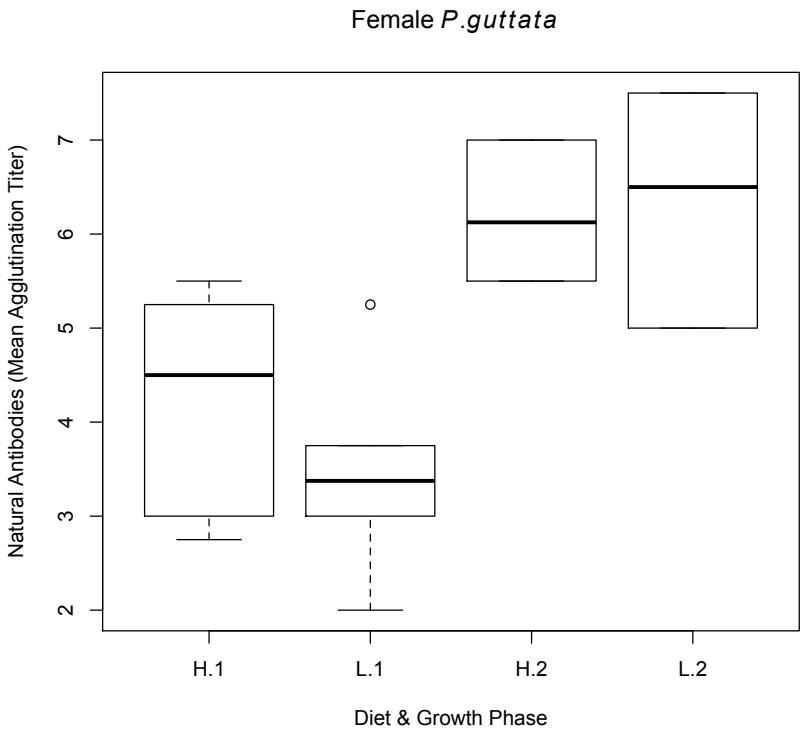

Male *T.marcianus*
Natural Antibodies (Mean Agglutination Titer)
7
6
5
4
H.1
L.1
H.2
L.2
Diet & Growth Phase

(a)

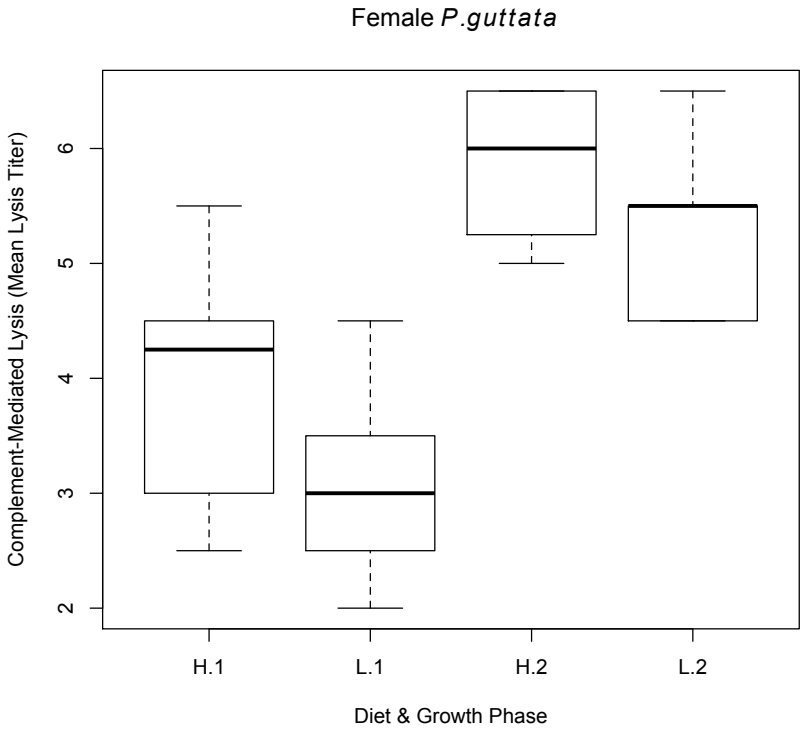

Male *T.marcianus*
Complement-Mediated Lysis (Mean Lysis Titer)
7
6
5
4
3
H.1
L.1
H.2
L.2
Diet & Growth Phase

(b)

Figure 9

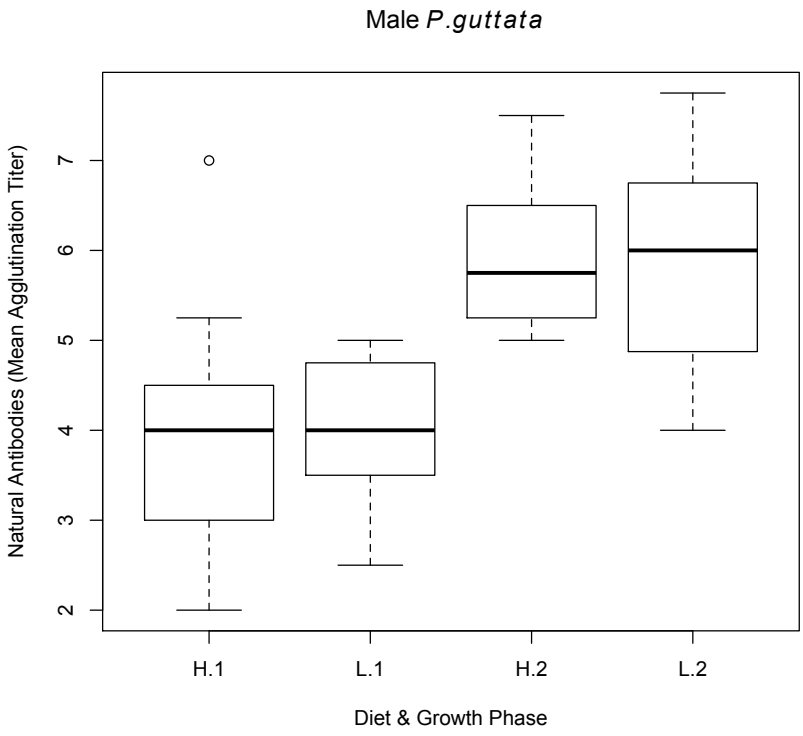

Female *P.guttata*
Natural Antibodies (Mean Agglutination Titer)
2
3
4
5
6
7
H.1
L.1
H.2
L.2
Diet & Growth Phase

(a)

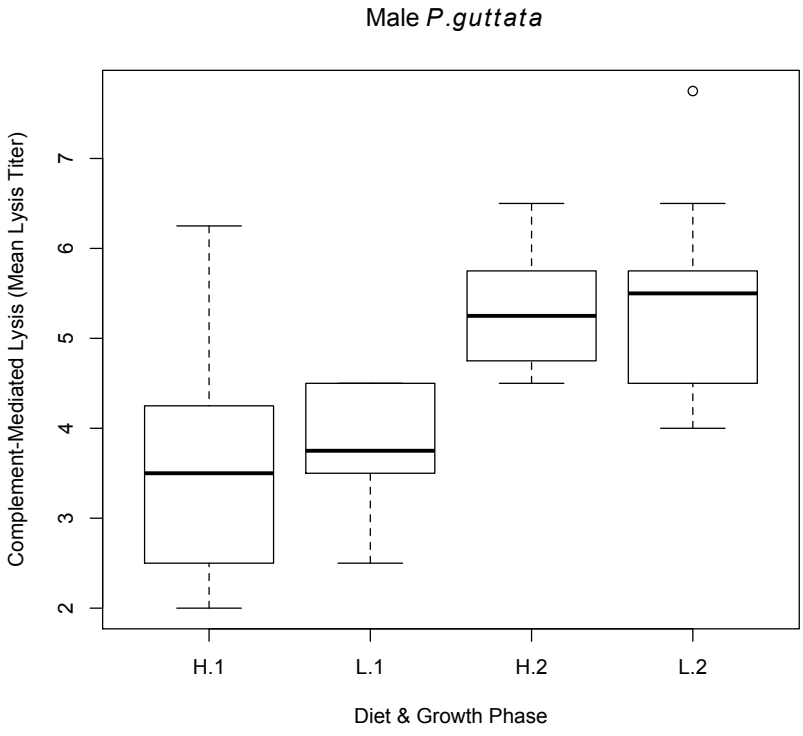

Female *P.guttata*
Complement-Mediated Lysis (Mean Lysis Titer)
2
3
4
5
6
H.1
L.1
H.2
L.2
Diet & Growth Phase

(b)

Figure 10

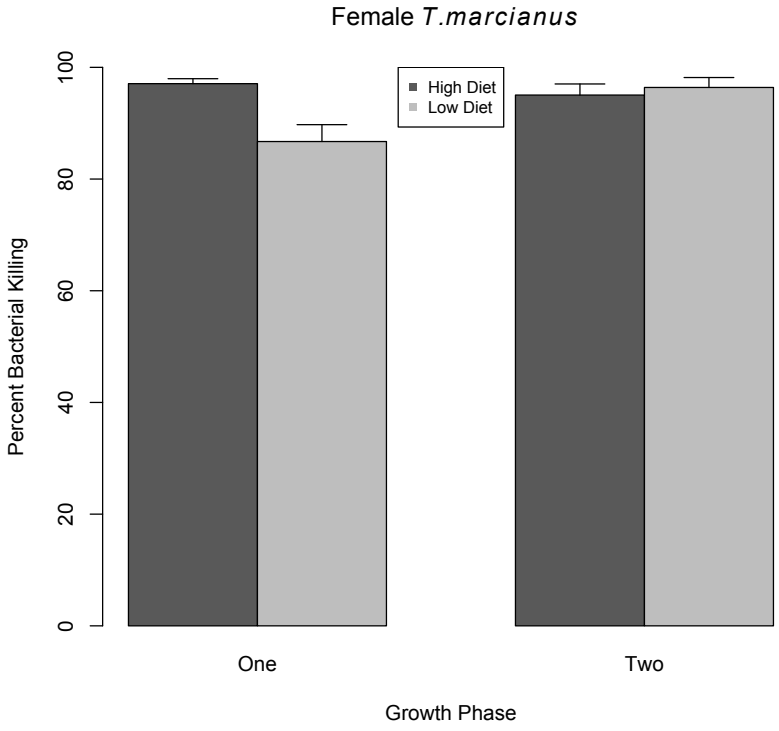

Male *P.guttata*
Natural Antibodies (Mean Agglutination Titer)
2
3
4
5
6
7
H.1
L.1
H.2
L.2
Diet & Growth Phase

(a)

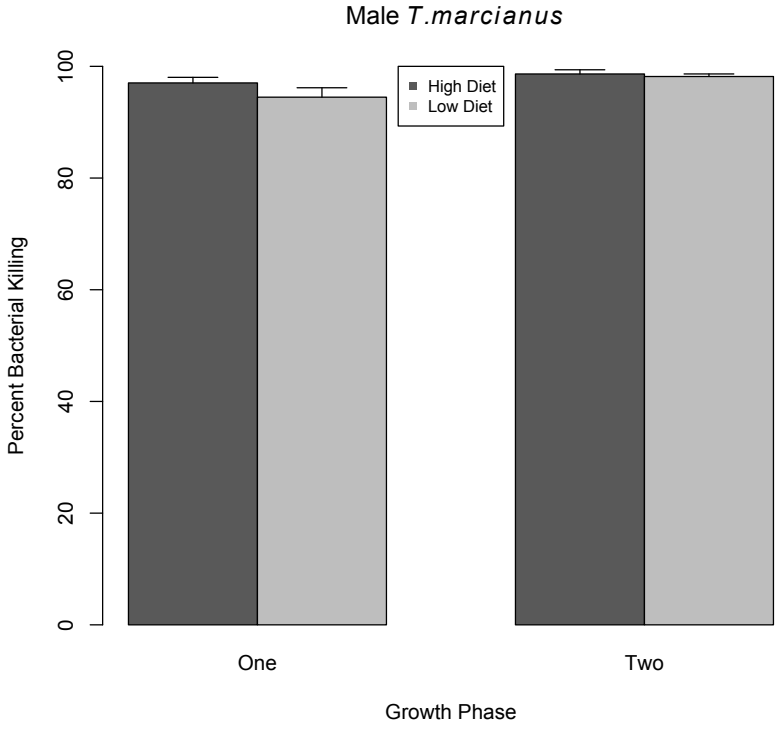

Male *P.guttata*
Complement-Mediated Lysis (Mean Lysis Titer)
2
3
4
5
6
7
H.1
L.1
H.2
L.2
Diet & Growth Phase

(b)

Figure 11

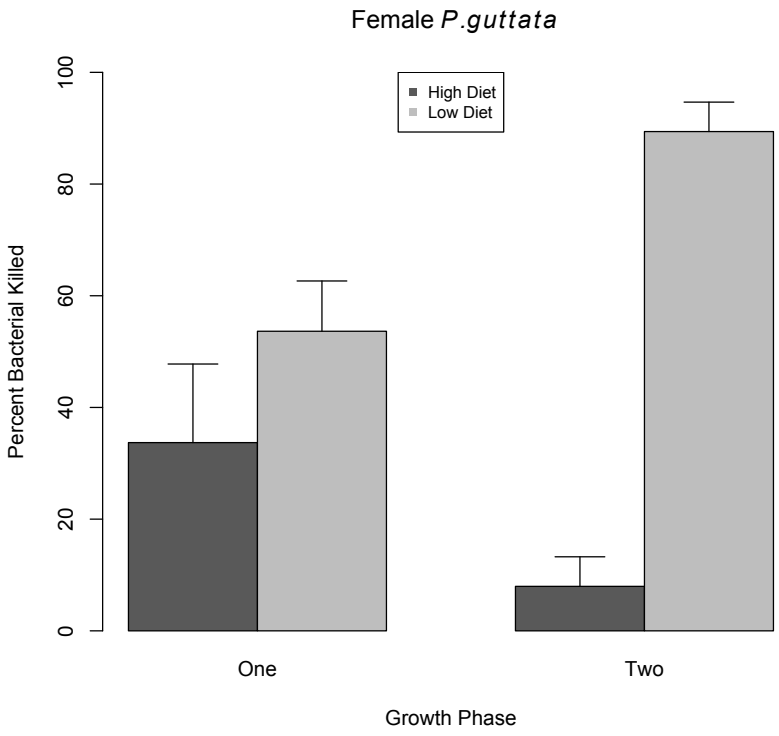

Female *T.marcianus*
High Diet
Low Diet
Percent Bacterial Killing
0
20
40
60
80
100
One
Two
Growth Phase

(a)

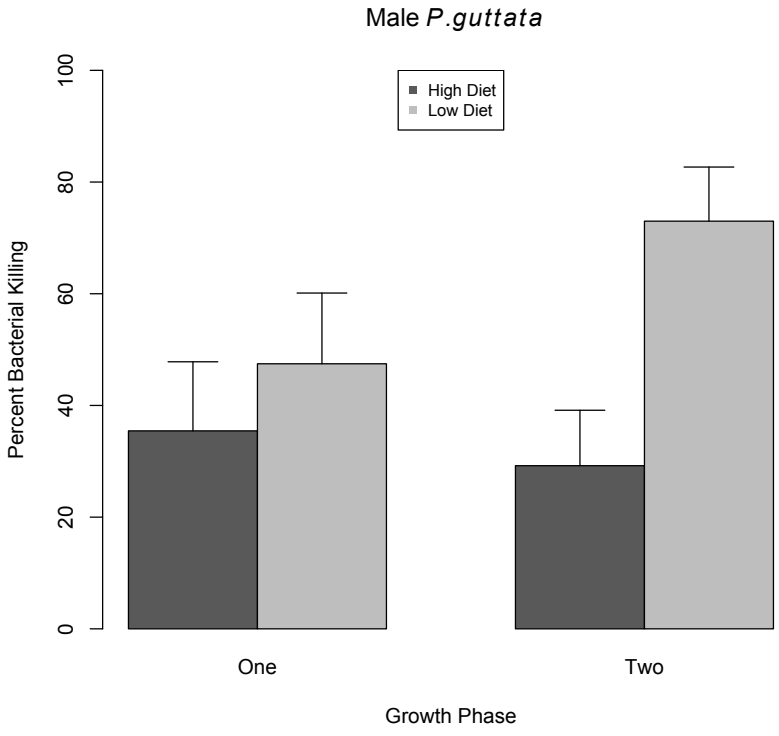

Male *T.marcianus*
High Diet
Low Diet
Percent Bacterial Killing
0
20
40
60
80
100
One
Two
Growth Phase

(b)

Figure 12

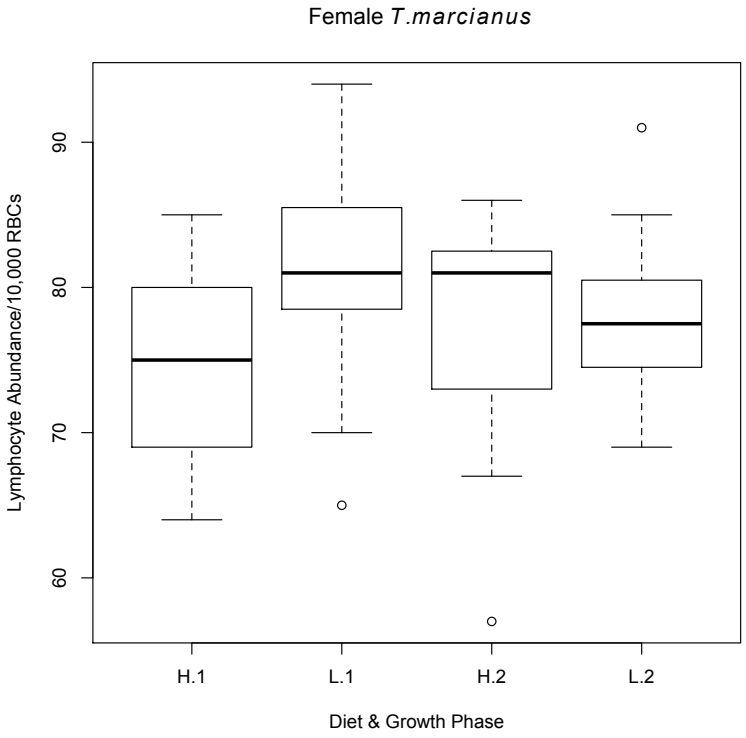

Female *P.guttata*
High Diet
Low Diet
Percent Bacterial Killed
0
20
40
60
80
100
One
Two
Growth Phase

(a)

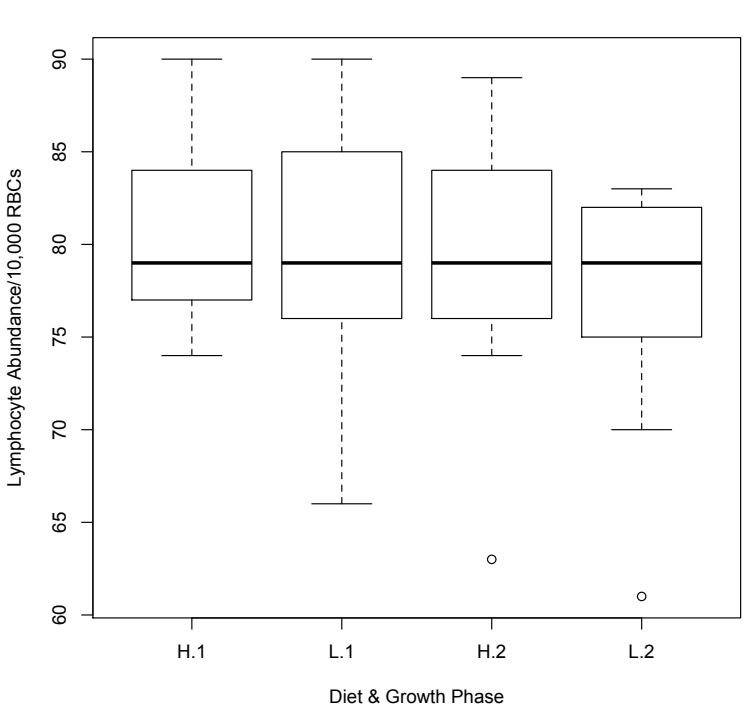

Male *P.guttata*
High Diet
Low Diet
Percent Bacterial Killing
0
20
40
60
80
100
One
Two
Growth Phase

(b)

Figure 13

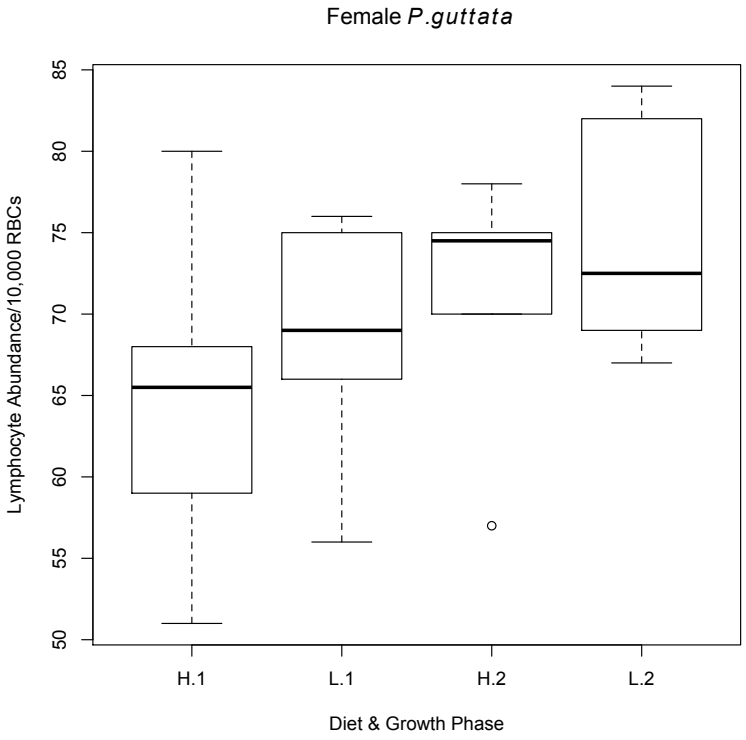

Female *T.marcianus*
Lymphocyte Abundance/10,000 RBCs
60
70
80
90
H.1
L.1
H.2
L.2
Diet & Growth Phase

(a)

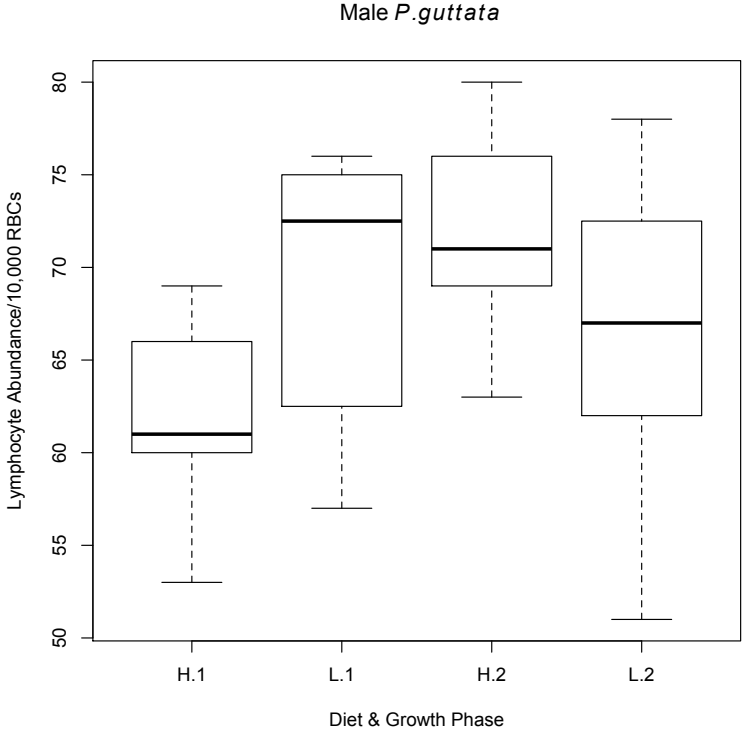

Male *T.marcianus*
Lymphocyte Abundance/10,000 RBCs
60
65
70
75
80
85
90
H.1
L.1
H.2
L.2
Diet & Growth Phase

(b)

Figure 14

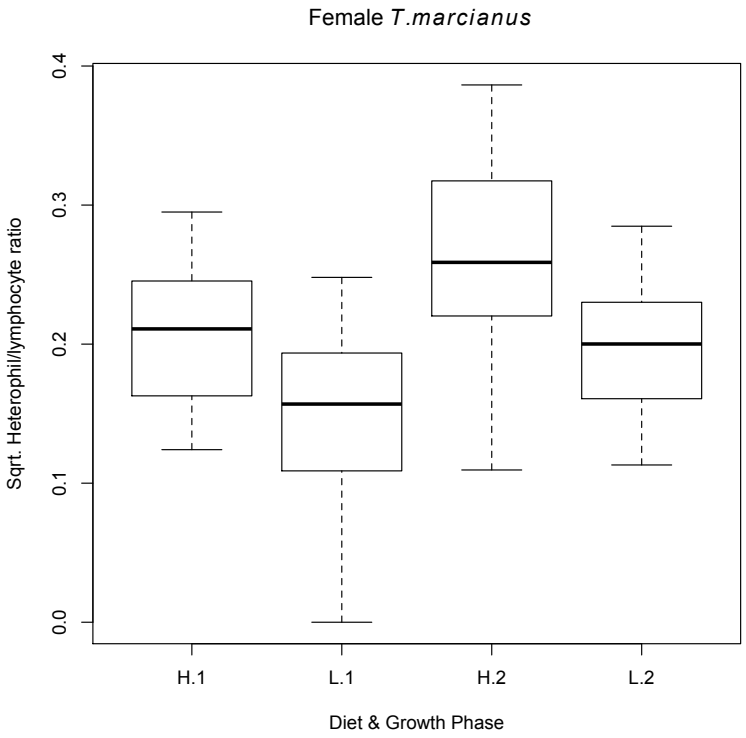

Female *P.guttata*
Lymphocyte Abundance/10,000 RBCs
50
55
60
65
70
75
80
85
H.1
L.1
H.2
L.2
Diet & Growth Phase

(a)

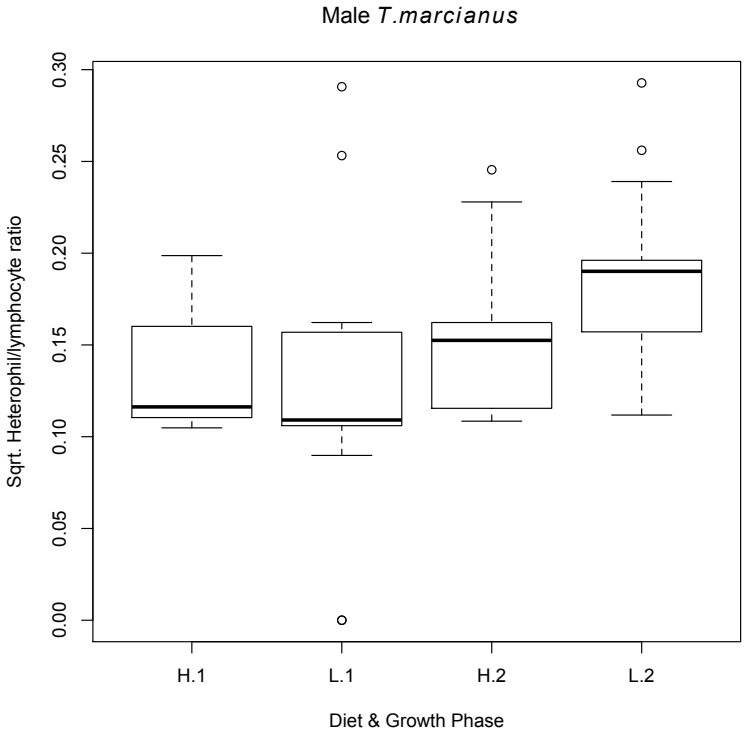

Male *P.guttata*
Lymphocyte Abundance/10,000 RBCs
50
55
60
65
70
75
80
H.1
L.1
H.2
L.2
Diet & Growth Phase

(b)

Figure 15

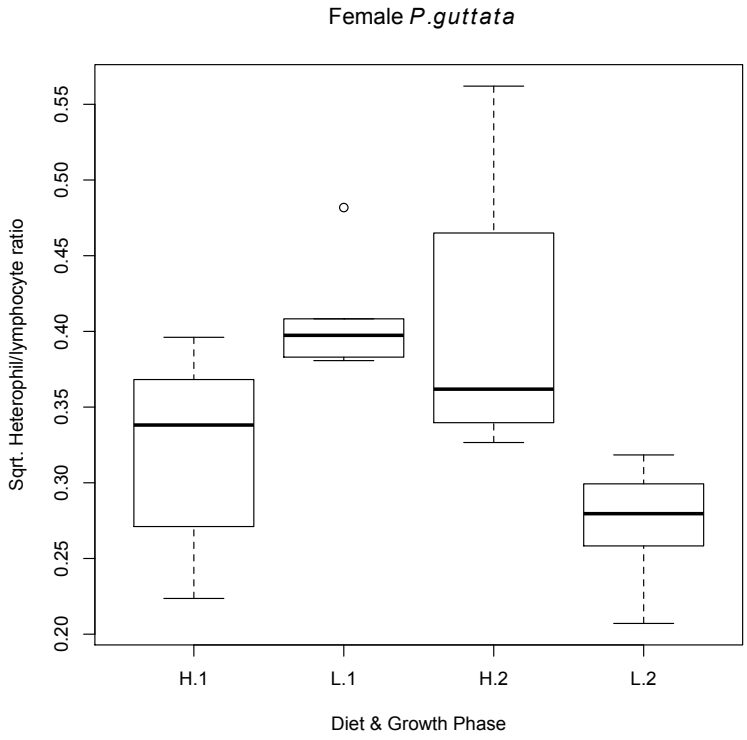

Female *T.marcianus*
Sqrt. Heterophil/lymphocyte ratio
0.0
0.1
0.2
0.3
0.4
H.1
L.1
H.2
L.2
Diet & Growth Phase

(a)

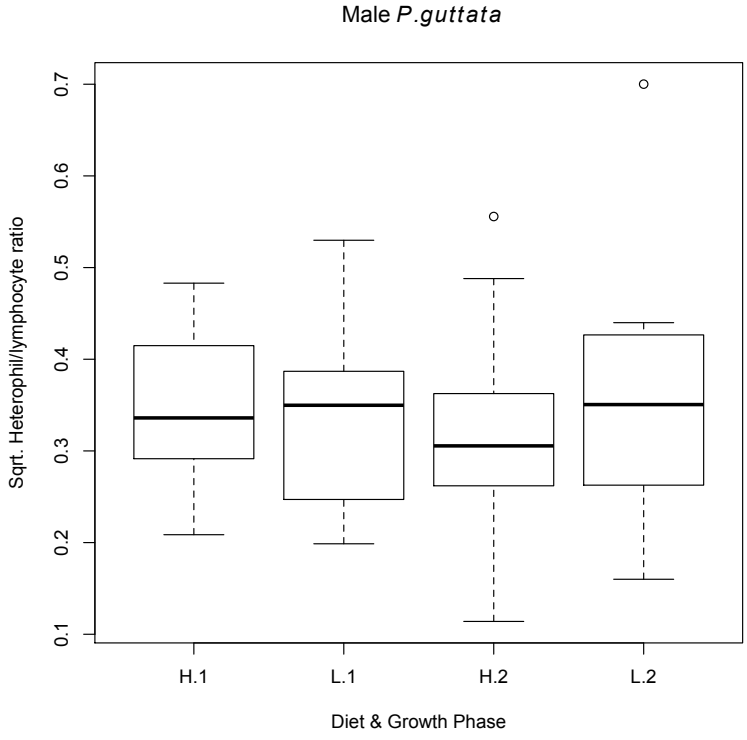

Male *T.marcianus*
Sqrt. Heterophil/lymphocyte ratio
0.00
0.05
0.10
0.15
0.20
0.25
0.30
H.1
L.1
H.2
L.2
Diet & Growth Phase

(b)

Figure 16


Female P.guttata
Sqrt. Heterophil/lymphocyte ratio
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0.55
H.1
L.1
H.2
L.2
Diet & Growth Phase

(a)


Male P.guttata
Sqrt. Heterophil/lymphocyte ratio
0.1
0.2
0.3
0.4
0.5
0.6
0.7
H.1
L.1
H.2
L.2
Diet & Growth Phase

(b)

CHAPTER FOUR

THE ROLE OF CORTICOSTERONE IN COMPENSATORY GROWTH IN THE CHECKERED GARTER SNAKE (*Thamnophis marcianus*)

INTRODUCTION

The term "compensatory growth" or "catch-up growth" describes both the pattern and the process by which an organism grows more quickly after a period of reduced growth than would be expected in the absence of restriction. In other words, compensatory growth is a phase of accelerated growth that occurs when normal nutrition is available after a period of growth inhibition (Ali et al, 2003; Metcalf and Monaghan, 2001). The accelerated growth is typically at a higher rate than expected in the absence of growth inhibition (Nicieza and Alvarez, 2009). Compensatory responses to growth restriction have been suggested to carry physiological (e.g. high metabolic rates that would more quickly deplete resources or allocate more resources into growth and less to energy storage), developmental (e.g. lower the degree of quality control with high probability for developing developmental errors), ecological (e.g. put an organism at a higher risk for predation) and immunological (e.g. decrease an individual's ability to defend against foreign agents or resist disease) costs (Gotthard, 2001).

Immune function is complex, and the relative contribution of different components (e.g. cellular vs. humoral) within species during periods of either homeostasis or stress is unclear (Matson et al., 2006). Relatively little is known about

the intra- or interspecific variation in immunological capabilities, especially in the context of the effects of stress on immune function (Matson et al., 2006). Upon exposure to unpredictable adverse events such as increased risk of predation, severe climatic conditions or food deprivation, the activity of the hypothalamic-pituitary-adrenal (HPA) axis (hypothalamic-pituitary-interrenal (HPI) axis in reptiles) (Greenberg and Wingfield, 1987) is up-regulated and secretion of glucocorticoids is increased (Palacios et al., 2012). Stress induced levels of glucocorticoids, corticosterone being the major glucocorticoid in reptiles (Greenberg and Wingfield, 1987), cause a shift in physiological and behavioral parameters such that self-maintenance and survival processes are prioritized (Palacios et al., 2012). By redirecting resources and behaviors, corticosterone and thus stress especially at chronic levels is generally considered immunosuppressive (Råberg et al., 1998). Thus it is suggested that stress, quantified through corticosterone levels, will be greater in animals that allocate excess energy into growth following a period of nutritional stress (phase of depressed growth).

METHODS

Compensatory growth studies with the checkered garter snake (*Thamnophis marcianus*) were previously conducted to determine the role of nutritional stress on growth rates when subsequent resources become plentiful. To determine the effects of nutritional stress and resulting compensation in growth on corticosterone levels, blood samples were collected from each animal. Blood samples of 50-250µl, depending on snake body size, were taken from the caudal vein of each animal using heparin-rinsed syringes. Handling time to time of completion of bleeding was recorded. After centrifugation, plasma was snap-frozen in liquid nitrogen and stored in an -80 freezer.

Blood samples were collected at the end of the first phase of growth (time of the diet shift), from each *T.marcianus* test subject (n=69) to test for physiological differences of stress based on diet restriction through testing levels of corticosterone. Allowing equal time for compensatory growth as was allotted for separation (105 days) blood was drawn a second time from each animal to test for the same physiological differences.

Corticosterone Assay

Corticosterone levels were quantified in plasma to control for its concentration at the time of blood sampling and to also determine its effect on immune response. Corticosterone levels were assessed with a radioimmunoassay (MP Biomedicals ImmuChem Double Antibody Corticosterone I-125 RIA kit, Irvine, CA) adapted for use with snakes. Because not all blood samples could be collected within three minutes of handling the animals, time between first contact and bleeding was included as a covariate in the statistical analyses of corticosterone levels.

Statistical Analysis

All analyses were conducted using R 2.15.3 statistical package (R Core Team, 2013). All data were checked for normality and were transformed as necessary. Data were separated by sex and analyzed for the effects of dietary treatment group and growth phase (representative of time and sample, first blood sample collected on day 105 and the second blood sample collected on day 210). Handling time was used as a covariate to correct for increased levels of corticosterone, as levels of glucocorticoids can be detected in circulation within three to five minutes of activation of the HPA axis.

RESULTS

Overall there was no difference between diet and growth phase on average levels of corticosterone for female *T.marcianus* (ANCOVA, $F_{1, 71} = 1.725$, $P = 0.193$). At the end of the first growth period, high diet and low diet treatment groups were indistinguishable from one another (ANCOVA, n = 37, $P = 0.172$; Fig 1a). The low diet treatment groups displayed a decrease in average H/L ratio during the second growth phase (ANCOVA, n = 34, $P = 0.636$ and n = 41, $P = 0.006$ for high diet and low diet treatment groups respectively; Fig 1a), however, at the end of the second growth phase the high diet treatment group and low diet treatment group had indistinguishable corticosterone levels (ANCOVA, n = 38, $P = 0.999$; Fig 1a).

There was no difference between diet and growth phase on average levels of corticosterone for male *T.*marcianus (ANCOVA, $F_{1, 56} = 3.508$, P = 0.066). At the end of the first growth period high diet and low diet treatment groups were indistinguishable from one another (ANCOVA, n = 30, $P = 0.255$; Fig. 1b). During the second growth phase neither treatment group displayed a significant change in corticosterone levels (ANCOVA, n = 30, $P = 0.999$ and n = 30, $P = 0.076$ for high diet and low diet treatments respectively; Fig 1b). Thus at the end of the second growth phase the two treatment groups were indistinguishable (ANCOVA, n = 30, $P = 0.886$; Fig 1b).

DISCUSSION

It has been found that exposure to unpredictable adverse events, such as food deprivation, causes an up-regulation of the hypothalamic-pituitary-interrenal axis, which in turn releases glucocorticoids in greater amounts (Palacios et al., 2012). It was therefore hypothesized that levels of corticosterone would be greater (up-regulated) in

low diet treatment animals for which compensation in size occurs after a period of natal stress (i.e. male checkered garter snakes). It was also hypothesized that increased levels of corticosterone would occur in the low diet animals (regardless the species and sex) that do not catch-up to their non-stressed conspecifics due to the chronic stress brought on by food deficits.

Female (Thamnophis marcianus)

At the end of the first growth phase there was no difference between the high and low dietary treatment groups for female *T.marcianus* ($P = 0.172$). During the second growth phase, only the low diet treatment group increased their corticosterone levels by a significant margin. However, at the end of the second growth phase there remained no difference between the two treatment groups ($P = 0.999$). These data suggest that nutritional stress and/or energy allocation into increased growth rates have little effect on stress levels through corticosterone.

Male (Thamnophis marcianus)

At the end of the first growth phase there was no difference between the high and low dietary treatment groups for male *T.marcianus* ($P = 0.255$) and neither group significantly increased or decreased their corticosterone levels during the second growth phase, and thus at the end of the second growth phase there remained no difference between the two treatment groups ($P = 0.886$). These data suggest that nutritional stress and/or energy allocation into increased growth rates cause little effect on stress levels through corticosterone.

Summary

These results differed from what was expected. Animals, which were subject to periods of nutritional stress (low food availability), did not show differences in corticosterone from the non-stressed conspecifics nor did their levels of corticosterone change with increased food availability. I suggest that this occurred either because snakes are robust in that they can maintain health at low levels of food intake as they often experience low prey availability and this may not trigger a stress response, or these animals responded to the stress by slowing their metabolic processes which in turn does not trigger the release of corticosterone.

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Råberg L., Grahn, M., Hasselquist, D. and Svensson, E. 1998. On the adaptive significance of stress-induced immunosuppression. Proceedings of the Royal Society of London B: Biological Sciences 265:1637–1641.

TABLES & FIGURES

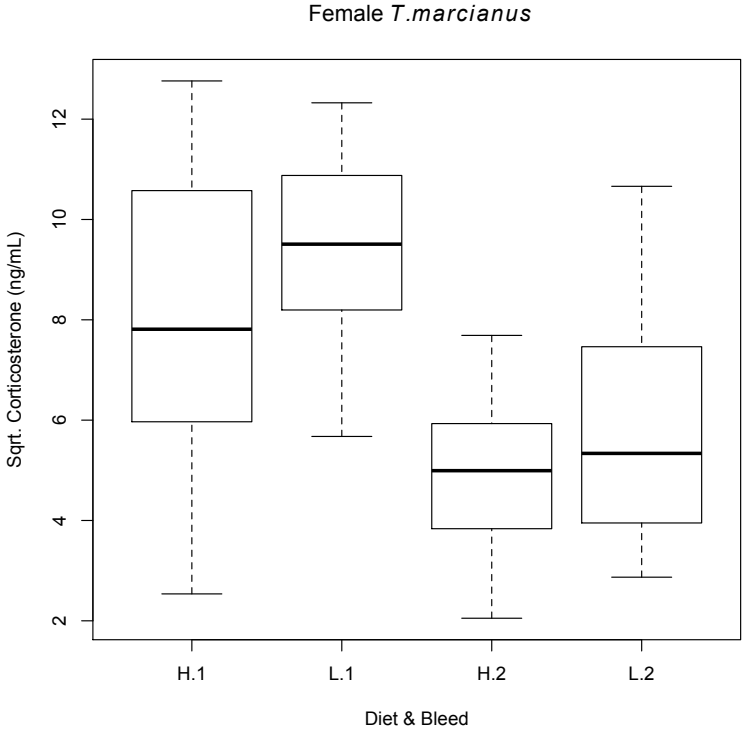
Table 9. Corticosterone levels (ng/mL) in peripheral blood between two dietary phases for female and male *Thamnophis marcianus*. Data represent raw numbers, stress of handling increases circulating levels of corticosterone and thus all statistics were run with handling time as a covariate. P-value obtained from Analysis of Covariance between dietary groups for each growth phase.

T.marcianus	Growth Phase 1 (day 105)						Growth Phase 2 (Day 210)					
	High Diet			Low Diet			High Diet			Low Diet		
Immune component (units)	Mean ± SE	min-max	n	Mean ± SE	min-max	n	Mean ± SE	min-max	n	Mean ± SE	min-max	n
Female Corticosterone (ng/mL)	66.69 ± 10.28	6.43-125.03	17	93.67 ± 7.45	32.21-151.92	20	32.50 ± 7.06	6.40-128.21	17	39.17 ± 6.51	8.23-113.66	22
	P = .172						*P* = .999					
Male Corticosterone (ng/mL)	66.01 ± 10.98	16.50-135.01	15	77.41 ± 13.56	19.02-197.73	15	52.46 ± 13.39	11.40-198.81	16	45.91 ± 11.61	7.03-143.66	15
	P = .254						*P* = .886					

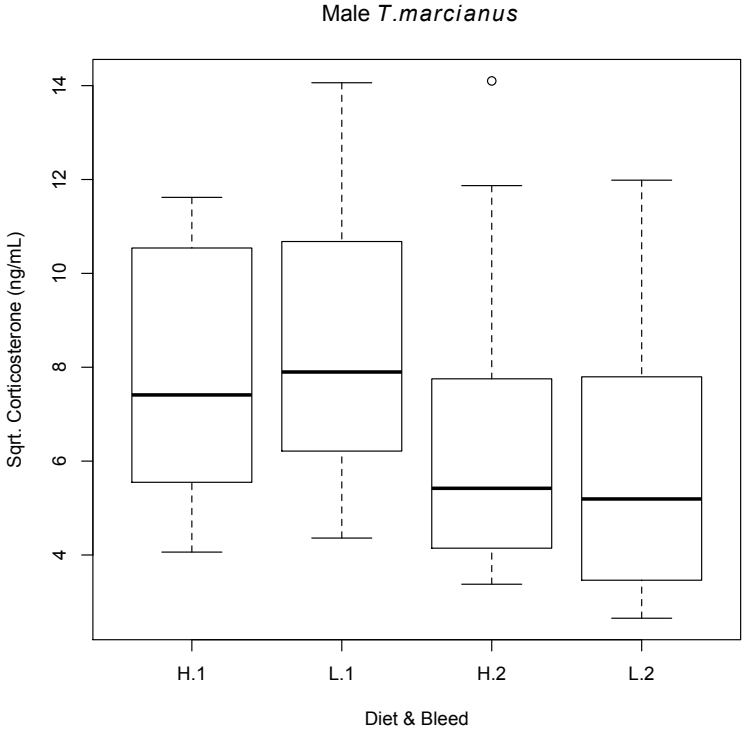
FIGURE LEGENDS

Figure 17. Comparison of corticosterone levels (ng/mL) for high and low diet treatment groups of (a) female *Thamnophis marcianus* and (b) male *Thamnophis marcianus* in the first growth phase (day 105, n = 37 and n = 30 for females and males respectively) and the second growth phase (day 210, n = 38 and n = 30 for females and males respectively). Box plots depict the median (bold horizontal line), interquartile range (height of box), range of observed values falling within a 3/2 spread of the interquartile range (whiskers), and the outliers (open circles).

Fig 17


Female *T.marcianus*
Sqrt. Corticosterone (ng/mL)
2
4
6
8
10
12
H.1
L.1
H.2
L.2
Diet & Bleed

(a)


Male *T.marcianus*
Sqrt. Corticosterone (ng/mL)
4
6
8
10
12
14
H.1
L.1
H.2
L.2
Diet & Bleed

(b)