INFLUENCE OF FOOD MANIPULATION ON PLASMA CORTICOSTERONE, INNATE IMMUNITY, AND BLOOD CHEMISTRY IN THE BROWN AFRICAN HOUSE SNAKE (*LAMPROPHIS FULIGINOSUS*)

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ABSTRACT

The ability to tolerate the stress of extended periods of food restriction is an evolutionarily important trait that has significant effects on survivorship in the wild. There are different physiological mechanisms by which certain species survive these periods: elevation of the stress hormone corticosterone, reduction of immune function, and reduction in reproductive activity are well documented responses to food restriction. These responses to food shortage have been documented in species ranging from mammals to amphibians, but to my knowledge, have not been performed within a sex-based context in colubrid snakes. Thus the objectives of this project are to document the effects of food manipulation and sex on plasma corticosterone, innate immunity and blood chemistry in the African brown house snake, *Lamprophis fuliginosus*. Sex differences would seem especially likely in reptile species that are strongly sexually dimorphic, such as the African brown house snake. With their larger body mass, females would be expected to have the capacity for much larger energy reserves than males and thus experience less dramatic changes in physiology in response to food restriction. This project consisted of a ten week food restriction period in which twenty-four snakes (16 female, 8 male) were fed just 5% of their body mass every two weeks and a five week recuperation period during which all snakes were fed ad lib. During the 15week project, four blood samples were collected from each snake to measure corticosterone, bacterial killing capacity (with *E. coli*), hemolytic capacity (with sheep RBC), leukocytes, testosterone, estradiol, triglyceride, and uric acid concentrations. I predicted that corticosterone would increase during the 10-week period of food restriction and that as

corticosterone increases, immune function would decrease with varying degrees between the sexes.

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CHAPTER I
INTRODUCTION

Strategies to survive food restriction

The ability to tolerate the stress of extended periods of food restriction is an evolutionarily important trait that has significant effects on survivorship in the wild (Shine and Downes, 1999). The physiological mechanisms by which certain species survive these periods fall into two basic categories: reductions in energy expenditure, and maximization of energy storage prior to the period of food restriction. Strategies to reduce energy consumption include behavioral modifications such as reductions in activity levels, reproduction, and mating behaviors. Physiological modifications include the suppression of the reproductive and immune systems and of the metabolic rate (Wingfield *et al*., 1998). Modifications can be both behavioral and physiological in nature: food restriction has been shown to influence the selected body temperature in both reptiles and fish (Sogard and Olla, 1996; Brown and Griffin, 2005). In the lizard, *Anolis carolinensis*, animals that were food deprived selected lower habitat temperatures. Similarly, in the fish, *Theragra chalcogramma*, starved animals selected colder water temperatures (Sogard and Olla, 1996). This lower body temperature may facilitate a lower metabolic rate (Sogard and Olla, 1996; Brown and Griffin, 2005). When the reduction of energy expenditures is not a viable possibility, the utilization of stored energy via lipolysis and other metabolic pathways results (Congdon, 1989).

The effect of stress on survival strategies has been studied in various species and under many contexts (Congdon, 1989; Sogard and Olla, 1996; Shine and Downes, 1999;

Brown and Griffin, 2005; Pough *et al*., 2004; French *et al*., 2007; Moore and Hopkins, 2009). In reptiles, the common factor covered in these studies is the role of the steroid hormone corticosterone.

Role of corticosterone

Corticosterone is a steroid hormone produced in the adrenal gland and is known to be elevated by stressors in reptiles (Dunlap, 1995; Moore and Jessop, 2003; Taylor and DeNardo, 2010). Its secretion is regulated by the hypothalamo-pituitary-adrenocortical (HPA) axis. The function of this stress hormone is context dependent and exhibits variation among species (Shine and Downes, 1999; Pough *et al*., 2004; French *et al.*, 2007; Moore and Hopkins, 2009). Documented functions of corticosterone vary from general energy mobilization to suppression or stimulation of the immune and reproductive systems (Shine and Downes, 1999; Moore and Jessop, 2003; Breuner *et al.*, 2008; Angelier and Wingfield, 2013). Moore and Jessop (2003) reviewed the HPA axis in amphibians and reptiles and highlighted the contextual dependence of the function of CORT between species. The general consensus in the literature is that during acute stress events, corticosterone mobilizes energy stores to supply critical organs with energy (i.e., muscles) (Dunlap, 1995; Moore and Jessop, 2003; Aldridge and Arackal, 2005; Taylor and DeNardo, 2010). During these events, corticosterone concurrently decreases the energy expenditures of unnecessary systems (i.e., energy storage, growth and reproduction). This inhibition of systems is context dependent: during active reproductive periods, corticosterone elevation in some species actually facilitates both physiological and behavioral changes necessary for successful reproduction (Moore and Jessop, 2003;

Lattin *et al*., 2012; Jaatinen *et al*., 2013). For example, in the Red-sided Garter snake (*Thamnophis sirtalis parietalis*), CORT is elevated in males during the spring breeding season. This elevation is thought to facilitate reproduction by mobilizing energy stores to sustain the costs associated with courtship and breeding (Moore and Jessop, 2003).

Effect of stress on reproduction and steroid hormones

Stress has been shown to suppress many aspects of reproduction in a diversity of vertebrates (Wingfield and Sapolsky, 2003). Elevations in CORT have been documented to terminate development of the sexual segment of the kidney and spermatogenesis in male snakes (Aldridge and Arackal, 2005). Similarly, in male wild brown tree snakes (*Boiga irregularis*), trapping and confinement lead to CORT elevation and the termination of spermatogenesis (Moore *et al*., 2005). In the Asp viper (*Vipera aspis*), females in poor body condition have high circulating CORT levels, low circulating estradiol concentrations, and are not sexually receptive. After body condition is improved, which is accompanied with a reduction in CORT levels, females exhibit elevated plasma estradiol and become sexually receptive (Aubret *et al*., 2002). Similarly, male *V. aspis,* with poor body condition, exhibited lower plasma testosterone and elevated CORT concentrations that inhibited courtship behaviors (Aubret *et al*., 2002). Snakes have a classic stress response, but the behavioral and or hormonal response may be tempered during periods of reproduction for species with limited reproductive opportunities (Sapolsky *et al*., 2000; Moore and Jessop 2003). In species with short breeding seasons, CORT elevation may not terminate reproduction. For example, *T. s. parietalis* has a very short breeding season and males captured during this time period

exhibit the typical CORT elevations but there is no change in courtship behavior (Moore *et al*., 2000).

Corticosterone's primary function as an energy mobilizing hormone complicates isolating CORT as an inhibitor of reproduction. The context dependent interaction between stress, corticosterone and reproduction has been the focus of several studies (Moore and Jessop, 2003; French *et al*., 2007). A positive relationship between reproduction and CORT has been regularly reported, which highlights the context-dependent physiological role that it plays. French *et al*. (2007) elaborated on the idea that moderately elevated levels of CORT facilitate reproduction via energy mobilization; whereas, extremely elevated CORT levels inhibit reproduction (Moore and Jessop, 2003).

Stress and immune function

"The role of stress in the etiology of diseases has been long studied: stress has been connected with immunosuppression and disease proliferation with a high level of consistency across species" (Dhabhar, 2002). The level or acuteness of the stressor has been shown to have a great deal of control on the physiological effects of stress. Acute stress, contrary to common thought, may actually act as an immunoenhancer: the initial elevation of glucocorticoids (GC) may help to prepare the immune system from pending challenges such as wound healing or infection (French *et al*., 2007). In this context, immunoenhancement would be evolutionarily advantageous (Dhabhar, 2002). However under chronic stress, immunosuppression is commonly documented (Dhabhar, 2002; French *et al*., 2006). Several studies have shown that stress reduces leukocyte proliferation in the blood stream (Salanitro and Minton, 1973; Dhabhar, 2002; Lutton and

Callard, 2006). The circulation of immune cells, from blood to various organs and then back to the blood stream, form an immune defense network (Dhabhar, 2002). The concentration of these cells is one measure of an individual's innate immunity and thus the activity level of the immune system. Leukocyte reduction in response to various stressors has been observed in various species and especially in reptiles (Dhabhar, 2002; French *et al*., 2006). It has been suggested that this drop in circulating leukocytes is an adaptive response and represents the redistribution of immune cells to the organs that are most likely to encounter antigens during a stressful event (Dhabhar and McEwen 1999; Dhabhar, 2002). This sequestering of white blood cells to these "first defense" areas (i.e., skin and lymph nodes) allows the strongest response to foreign material entering the body (Dhabhar and McEwen 1999; Dhabhar, 2002; Dhabhar, 2009). Glucocorticoids (GC), such as corticosterone in reptiles, may also have a direct effect on circulating lymphocytes due to the presence of GC receptors. These GC receptors expressed on immune cells bind CORT, which causes interference with the function of the NF-kB pathway. This pathway regulates the activity of cytokines produced by immune cells (Padgett and Glaser, 2003). The context dependent relationship between energy requirements and physiological processes has led many investigators to focus on stress and its effects on resource allocation (Dhabhar, 2002; French *et al*., 2006).

Sex differences in response to food restriction

Few studies have asked whether there are sex differences in physiological response to food restriction. Sex differences would seem especially likely in reptile species that are strongly sexually dimorphic, such as the African brown house snake (*Lamprophis fuliginosus*). Adult female *L. fuliginosus* have a body mass on average 3 to 4 times larger than that of adult males (Ford, 2001). With their larger body mass, females should have the capacity for much larger energy reserves than males and thus experience less dramatic changes in physiology in response to food restriction. Corticosterone, the primary glucocorticoid in snakes, plays a major role in energy mobilization. Glucocorticoids are important regulators of energy stores, including carbohydrates, lipids, and proteins. Elevated levels of CORT are generally observed during periods of stress and act to mobilize fat stores (Wingfield *et al*. 1998, Pough *et al*., 2004; French *et al.*, 2007; Moore and Hopkins, 2009). In general, animals in poor body condition are expected to have elevated circulating levels of CORT, but it has been proposed that the sexes may exhibit different relationships between body condition and CORT (Toft, 1985; Stoney *et al.*, 1988; Dunlap, 1995; Sogard and Olla, 1996; Moore and Jessop, 2003; Moore and Hopkins, 2009). This CORT and body condition relationship has been reported in several studies, but the effect of sex on this relationship has received little attention in snakes.

Study objectives

In this study the African brown house snake (*Lamprophis fuliginosus),* a strongly sexually dimorphic species, is used to examine the effects of food restriction on circulating levels of steroid hormones, immune function, and blood chemistry. With the

high energy requirements associated with reproduction, body condition is especially important in influencing the reproductive capacity of snakes (Pough *et al*., 2004). Many snakes use a capital breeding strategy rather than one based on income: capital breeders base reproductive activities on energy reserves more than on current food intake (Gregory, 2006). Capital breeding species are typically found in low energy habitats and have few reproductive events. Conversely, income breeding species are found in less harsh habitats and have multiple reproductive events (Doughty and Shine, 1997; Bonnet *et al*., 1998). The species used in this study, *Lamprophis fuliginosus,* is an income breeder based on the prolific nature of documented reproductive events (Walker and Ford, 1996; Ford, 2001; Byars *et al*., 2010). The African brown house snake can breed year round and produce a clutch every five weeks (up to 12 clutches per year) (Ford, 2001). Because of this year round reproductive strategy and multiple reproductive opportunities, I expect to observe especially sensitive and robust corticosterone responses to food restriction with pronounced decreases in innate immunity and decreases in the concentration of circulating reproductive hormones (testosterone and estradiol). In addition to testing for main effects of food restriction, I also test whether the percent body mass loss is associated with plasma CORT or immune measures. The allocation of energy for offspring production and self-maintenance can be physiologically taxing, especially during a resource limited period (Pough *et al*., 2004). Therefore, I expect percent mass loss to be positively correlated with plasma corticosterone concentration and negatively correlated with bacterial killing capacity, hemolytic capability, and leukocyte proliferation.

A second objective is to determine if the sexes differed in their responses. Previous studies have demonstrated that the stress response in reptiles after acute stress can be dependent on sex (Grassman and Hess, 1992; Jessop, 2001). Males are less able than females to modulate the adrenocortical responses, and they exhibit higher circulating CORT concentrations than reproductively active females (Jessop, 2001). Because male African brown house snakes are much smaller than females and thus have a smaller capacity for energy storage, they should exhibit a greater physiological response to 10 weeks of food restriction than females. Therefore, I expect to observe higher CORT levels and lower measures of innate immunity (i.e., leukocyte counts, bacterial killing and hemolytic capacity) in males than in females.

Plasma concentrations of triglycerides and uric acid are used in this study as measures of body condition and metabolic state. Acute elevations of glucocorticoids have been shown to increase lipolysis of plasma triglyceride (McCue, 2007; Harmon *et al*., 2011; Neuman-Lee *et al*., 2015), and the released fatty acids undergo gluconeogenesis in the liver (Pough *et al*., 2004). Consequently, compared to animals not under chronic stress, animals experiencing an extended period of food restriction should have lowered plasma triglyceride concentrations as fat is utilized to produce free glucose. Baseline CORT has been shown to be negatively correlated with triglyceride concentrations in female fence lizards (Phillips and Klukowski, 2008). Uric acid is a nitrogenous waste produced in reptiles from the catabolism of protein (Totzke *et al*., 1999; Pough *et al*., 2004). In response to food restriction, large spikes in uric acid concentrations should be seen once body condition has deteriorated to the point of muscle catabolism (Totzke *et al*., 1999).

CHAPTER II

MATERIALS AND METHODS

Design and overview of project

Adult male and female African brown house snakes, *Lamprophis fuliginosus*, were used in this study to evaluate any potential interactions between food restriction stress, innate immunity, steroid hormones, body condition, and sex. The snakes were acclimated to laboratory conditions for 28 days prior to initiation of the food restriction. At the initiation of the 15 week study, blood was collected from each snake to establish a baseline measurement of hormones, immune function and blood chemistry. Subsequent blood samples were collected at weeks 5 and 10 of food restriction during which snakes were fed meals 5% of their body mass every other week. Upon conclusion of the 10 week period of food restriction, snakes were fed *ad lib* weekly for the final 5-week recuperation phase after which a fourth and final blood sample was collected.

Study species

The African brown house snake, *Lamprophis fuliginosus*, is a medium sized colubrid that is endemic to southern Africa (Ford, 2001; Byars *et al*. 2010). The total length range of a female is 90-120 cm and a male is 45-70 cm. This species is a nocturnal, generalist feeder and lives well in a laboratory setting. Twenty-four African house snakes (16 female and 8 male) were obtained on loan from Dr. Neil Ford, director of the Ophidian Research Colony of the University of Texas at Tyler. Individuals snakes were housed individually in plastic cages (60 cm x 40 cm x 15 cm) lined with aspen

substrate and fed commercially purchased mice. The subjects were acclimated to laboratory conditions for 28 days and were fed ~20% of their body mass twice weekly prior to the initiation of the food restriction. The housing temperature was maintained at 27°C with a 12:12 light ratio. These housing conditions are similar to those successfully used by Dr. Neil Ford (Ford, 2001).

Feeding and handling

Every 2 weeks, during the 10 week food restriction phase, all animals were fed mice weighing approximately 5% of their most recently recorded body mass. After the 10 week sampling period, snakes were fed *ad lib* every week for the final 5 weeks of the study. To minimize extraneous stress, the animals were not handled except while cleaning cages and collecting data. During data collection periods, the animals were removed from their containers in a particular order that was maintained throughout the study to control for circadian rhythms within individual snakes. In each case, data collection began at 0845 hours and concluded at 1400 hours. To control for a potential sex bias attributed to the order in which each sex was sampled, a pattern was established in which 2 females and then 1 male were bled sequentially.

Blood collection and processing

Blood collection began immediately after animals were removed from their cages. Bleed time did not exceed 5 minutes and averaged 3.9 ± 0.95 minutes (n = 94). Blood was collected by piercing the palatine vein in the oral cavity with a 27 ga needle and collecting the upwelling blood into heparinized microcapillary tubes. If blood was not

able to be collected from the mouth, then it was obtained via tail clipping (n = 14). Blood smears were made for each individual immediately after blood collection (see below) and the remaining blood was then centrifuged. The hematocrit was measured and plasma removed using a 100-µl Hamilton gas chromatography syringe (cleaned with deionized water between each animal). The collected plasma was then aliquotted into Eppendorf tubes to prevent repeated freeze-thaw cycles and stored at -80ºC. After blood had been collected, body mass, snout-vent length (SVL), and tail length (TL) were measured for each animal. Body mass was determined to the nearest 0.01 g and length measurements were obtained to the nearest 0.5 cm.

Measures of innate immunity

Bacterial killing capacity

The bacterial killing capacity of plasma gives a standard measure of innate immune function via the ability of a host to eliminate a foreign pathogen (Ruiz *et al*., 2010). Thirty µl of 11X diluted plasma (12 µl of plasma and 120 µl of PBS) and 10 µl of *E. coli* (1 x 10^5 /ml) were added in quadruplicate to a 96-well plate and then mixed on a plate shaker. To initiate the killing stage of the assay, the plate was incubated for 30 minutes at 27ºC (preferred temperature of the study species). To end this killing stage, 125 µl of tryptic soy broth was added to each well. The absorbance value, for this initial stage, was determined with a microplate reader at 340 nm. After a 12 hour incubation period at 37ºC (preferred temperature of *E. coli*), a final absorbance value was determined after vigorous mixing to break up any clumped *E. coli*. Negative and positive controls were run on each plate: positive controls contained 40 µl of PBS in place of

snake plasma and negative controls contained 30 µl of PBS without *E. coli*. The bacterial killing capacity value was determined as: 100[1- Δ sample/ Δ positive control].

Hemolysis

Hemolysis of sheep red blood cells exposed to snake plasma is caused by the complement proteins and natural antibodies (Nabs) present in plasma (Merchant and Britton, 2006). To measure the innate ability of the plasma to lyse foreign cells *in vitro*, plasma from each individual was mixed with PBS (0.01M) and sheep red blood cells (Lampire, #7209003) that had been washed in Dulbecco's PBS. In brief, 15 µl of 0.01M PBS was added to columns 1-11 of a 96-well plate, then 15 µl plasma was added to the first column and serially diluted by aspirating and ejecting from column to column. Then 15 µl of 1% sheep red blood cell suspension was added to each well. The plate was incubated at 27º C for 90 minutes and then an additional 60 minutes at room temperature (to allow maximum lytic activity). After maximum lytic activity was complete, the well at which lysis ceased was recorded for each individual using the control wells as a reference. Positive controls contained sheep red blood cells and 15 µl of deionized water; negative controls contained sheep red blood cells and 15 µl of PBS. Lysis was determined by the coloration of each well: lysed cells dissipated within the PBS giving the well a pink coloration; whereas, unlysed cells settled to the bottom of the well and formed a visible red pellet. Titers were estimated as the negative $\log_2$ of the highest dilution factor of plasma that showed lysis.

Leukocytes

Blood smears were air dried and then fixed in methanol before being stained with Giemsa (J.T. Baker Co.) at a later date. Baseline and 10 week blood smears were examined at 600X magnification and numbers of leukocytes recorded, giving special attention to heterophils and lymphocytes. Heterophils are the largest of the leukocytes, have a round shape, and an eccentric nucleus. Lymphocytes are characterized by a dark staining nucleus that fills most of the cell and is surrounded by a thin rim of cytoplasm that is blue in coloration.

Total red blood cells were counted using ImageJ (http://rsb.info.nih.gov/ij/) at 600X. We report the total leukocytes per 10,000 RBC, heterophils per 10,000 RBC, and the heterophil:lymphocyte ratio (H:L). Because of a large number of densely packed blood smears, only 8 snakes had readable blood smears for both the baseline and 10 week periods (females, n = 4 and male, n = 4).

Plasma hormone assays

Corticosterone

Baseline plasma corticosterone (CORT) concentrations were quantified via commercially purchased enzyme-linked immunosorbant assay kits (Corticosterone EIA kit ADI-900-097, Enzo Life science). Plasma from each snake was thawed for 20 minutes at 25ºC and then thoroughly mixed. Twenty µl of the prepped plasma was then diluted with 20 µl of steroid displacement reagent, mixed, and allowed to sit for at least 10 minutes. Assay buffer was then added (760 µl) to each sample to bring the total dilution of each sample to 40X. All samples were run in duplicate. The polyclonal antibody

provided a competitive binding environment between the CORT standards and the endogenous CORT in the plasma samples. After a 2 hour incubation period at room temperature the plates were washed three times. After addition of pNpp (p-nitrophenyl phosphate) substrate and a 1 hour incubation at 25ºC, the color development was quantified in a microplate reader at 405 nm. Absorbance values of the known concentrations in the standards provided a reference for the unknown concentrations found in the plasma samples tested. The intraassay coefficient of variation was 7% and the interassay coefficient of variation was 14%.

Estradiol and testosterone

Testosterone and 17β estradiol (E_2), reproductive hormones known to be affected by stressors and elevated plasma corticosterone (Grassman and Hess, 1992; Moore *et al*., 2000; Moore and Jessop, 2003), were quantified via commercially purchased enzyme-linked immunosorbant assay kits (Testosterone EIA kit ADI-900-065 and 17β Estradiol high sensitivity ELISA kit ADI-900-174, Enzo Life science). Plasma (20 µl) was treated with steroid displacement reagent as described above and then diluted in assay buffer to 30X for testosterone or 25X for estradiol. The testosterone assay used a monoclonal antibody and conversely the E_2 assay used a polyclonal antibody. After the specified antibody was introduced to the sample and standards, testosterone plates were incubated for 1 hour and E_2 plates for 2 hours. Conjugate was then added to the testosterone assay followed by another 1 hour incubation period (E_2 assay mixed antibody and conjugate prior to the 2 hour incubation). The intraassay coefficient of variation was 5% for

testosterone and 10% for estradiol. Because each assay required only one plate, there is no interassay coefficient of variation.

Measures of body condition

Percent body mass loss

Percent body mass loss was calculated by dividing the grams lost over the 10 week period of food restriction by the baseline body mass. The subsequent percentage was then arcsine transformed.

Triglyceride assay

Plasma triglyceride levels were measured with a commercially purchased enzyme kit (Triglyceride reagent no. T7532, Pointe Scientific Inc.). The triglyceride assay used an enzymatic reaction involving the conversion of triglyceride to quinoneimine dye. In brief, triglyceride is hydrolyzed by lipase into glycerol which is then converted by glycerol kinase into Glycerol-1-phosphate. This product is then oxidized by glycerol phosphate oxidase (GPO) and produces hydrogen peroxide (H_2O_2). When H_2O_2 is mixed with 4-chloropheno and 4-aminophenazone (4-AA) and peroxidase (POD) it produces quinoneimine dye. The intensity of this red colored dye is directly related to the concentration of triglyceride within the sample. The concentration is quantified via spectrophotometry at 500 nm and comparison to a triglyceride standard. In brief, 10 µl of plasma was mixed with 1.0 ml of warmed reagent and then incubated for 20 minutes at 37º C. Plasma triglyceride concentrations were measured in mg/dL.

Uric acid assay

Uric acid was also measured with a commercially purchased enzyme kit (Uric acid reagent no. U7581, Pointe Scientific Inc.). The uric acid assay involved an enzymatic reaction involving uric acid, O_2, water, and uricase to produce allantoin, CO_2, and H_2O_2. The H_2O_2 produced was then mixed with 4-aminoantipyrine (4-AAP) and 2-Hydroxy-3,5-Dichloro-benzenesulfonate (HDCBS) and POD to form chromagen. Chromagen, a red dye, is directly related to the concentration of uric acid within the sample. The uric acid concentration was quantified via a microplate reader at 550nm and comparison to a uric acid standard. In brief, 10µL of plasma was mixed with 325µL of warm reagent and then incubated for 10 minutes at 37ºC. The concentration of uric acid was measured in mg/dL.

Statistical analyses

A repeated measures ANOVA with a between subjects factor of sex and a within subjects factor of time (e.g., sampling period) was used to determine the effect of food restriction on SVL, body mass, corticosterone concentration, and bacterial killing capacity. Paired t-tests were used to determine the effect of 10 weeks of food restriction on leukocyte counts. Because of small sample sizes, the nonparametric Wilcoxon test was used to test for an effect of food manipulation on plasma estradiol in females and testosterone in males. A Friedman two-way analysis of related samples was used to determine the effect of food restriction on hemolysis titer, plasma triglyceride and plasma uric acid concentration. Spearman rho correlation tests were used to determine whether the percent loss in body mass over the 10 weeks of restricted feeding was correlated with

corticosterone, bacterial killing, hemolysis, or leukocyte counts. Because the equal variance requirement of parametric statistics was not achieved with all time periods, nonparametric tests were sometimes used.

CHAPTER III
RESULTS

Effect of restricted feeding on body size

There was an effect of food restriction and refeeding on the body mass of snakes (RM ANOVA $F_{3,\ 60}$= 11.89, p = 0.001, Table 1). Sex also had an effect on body mass ($F_{1,\ 20}$= 23.88, p = 0.003), and there was an interaction between food restriction and sex ($F_{3,\ 60}$= 5.24, p = 0.001). In females, the mean loss during the 10 week period of restricted feeding was 12.9 ± 7.4% and in males the mean loss was 13.7 ± 8.1%. The mean body mass of females was 276.4 ± 22.0g whereas the mean for males was 70.2 ± 36.0g. Sidak corrected pairwise comparisons indicated that body mass after 10 weeks of food restriction was significantly lower than body mass at baseline (p = 0.001), week 5 (p = 0.001), or week 15 (p = 0.001). There was no significant difference between baseline and week 15 (p = 0.66).

There was no effect of food restriction and refeeding on the snout-vent length of snakes (RM ANOVA $F_{3,\ 60}$= 1.78, p = 0.16, Table 1). Sex had a significant effect on SVL ($F_{1,\ 20}$ = 48.40, p = 0.001), but there was no significant interaction between food restriction and sex ($F_{3,\ 60}$= 1.56, p = 0.21, Table 1). At the initiation of the *ad lib* feeding period, two male subjects died presumably because of their inability to withstand chronic food restriction. These males exhibited the largest percent body mass losses of the study (20% and 32% lost, respectively, by week 10).

Table 1.

Mean (± SD) body mass and snout-vent length of female and male Lamprophis fuliginosus.

		Baseline		5 wk restricted		10 wk restricted		15 wk refed	
Mass (g)	Females	292.39	(102.05)	276.86	(99.55)	256.47	(101.19)	279.86	(101.98)
	Males	71.98	(27.12)	69.72	(26.66)	65.35	(25.25)	73.60	(28.28)
SVL (cm)	Females	88.34	(11.25)	89.83	(11.37)	89.53	(12.50)	89.41	(11.58)
	Males	53.78	(6.53)	53.65	(6.23)	53.82	(6.89)	54.75	(7.01)

Note: Mean (± SD) body mass and snout-vent length of female (n = 16) and male (n = 8, but for week 15, n= 6 since two males died) *Lamprophis fuliginosus*. Snakes were fed 5% of their body mass every two weeks during the 10 week period of food restriction. After the 10 week sampling period, snakes were fed *ad lib* every week for the final 5 weeks of the study.

Steroid hormones

Corticosterone

There was not a significant effect of food restriction or refeeding (RM ANOVA $F_{3,\ 60}$= 0.79, p = 0.51), sex ($F_{1,\ 20}$= 0.59, p = 0.45), or an interaction between food restriction and sex ($F_{3,\ 60}$= 0.08, p = 0.63) (Fig. 1). The mean plasma corticosterone concentration for females was 100.3 ± 108.2 ng/mL; whereas, the mean for males was 324.9 ± 770.3 ng/mL. The two males that died produced the highest concentrations of CORT measured in this study (after 10 weeks of food restriction, plasma concentrations of 486.2 ng/mL and 4000.0 ng/mL).

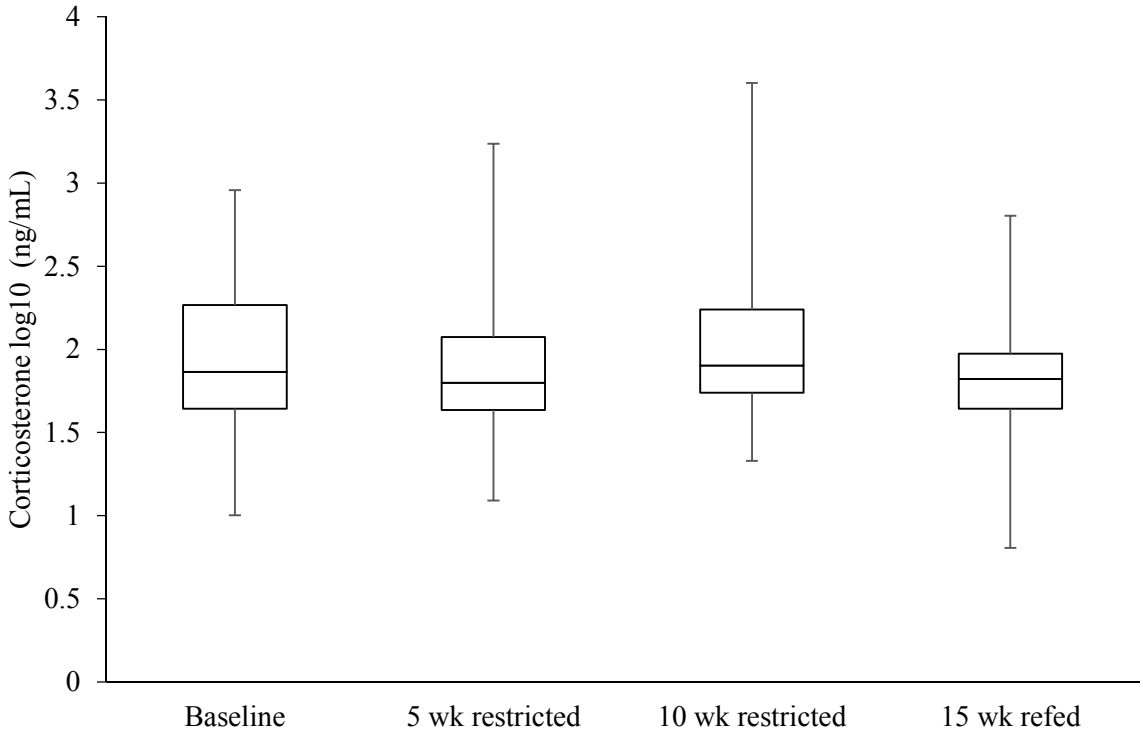

Figure 1. Changes in circulating corticosterone concentrations in female and male *Lamprophis fuliginosus*. Snakes were fed 5% of their body mass every two weeks during the 10 week period of food restriction. After the 10 week sampling period, snakes were fed *ad lib* every week for the final 5 weeks of the study (n = 24, 24, 24, 22). The box plots indicate the median, upper and lower quartiles, and the range of values.

Estradiol

There was not a significant effect of 10 weeks of food restriction on plasma estradiol in females (W = - 1.15, p = 0.25, Fig. 2). Similarly, estradiol levels in females after 5 weeks of *ad lib* feedings were not significantly different from levels at 10 weeks (W = -0.13, p = 0.89). The mean plasma estradiol concentration for females was 38.6 ± 82.7 pg/mL.

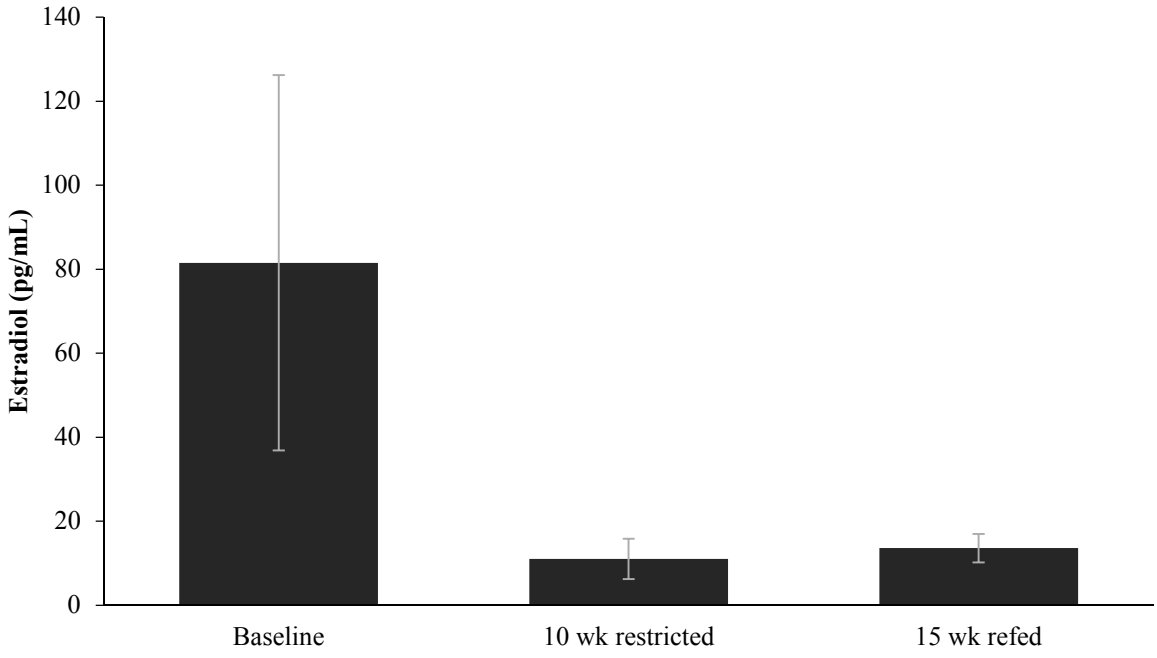

Figure 2. Mean (±SE) circulating estradiol concentrations in female *Lamprophis fuliginosus.* (n = 8, 7, 6, respectively).

Testosterone

There was not a significant effect of 5 weeks of food restriction (5 wk vs baseline, $W = -0.31$, $p = 0.75$) or 10 weeks (10 wk vs baseline, $W = -0.73$, $p = 0.46$, Fig. 3) on plasma testosterone. Similarly, testosterone levels in males after 5 weeks of *ad lib* feedings were not significantly different (15 wk vs 10 wk $W = -1.34$, $p = 0.18$). The mean plasma testosterone concentration for males was 9.43 ± 2.06 ng/mL.

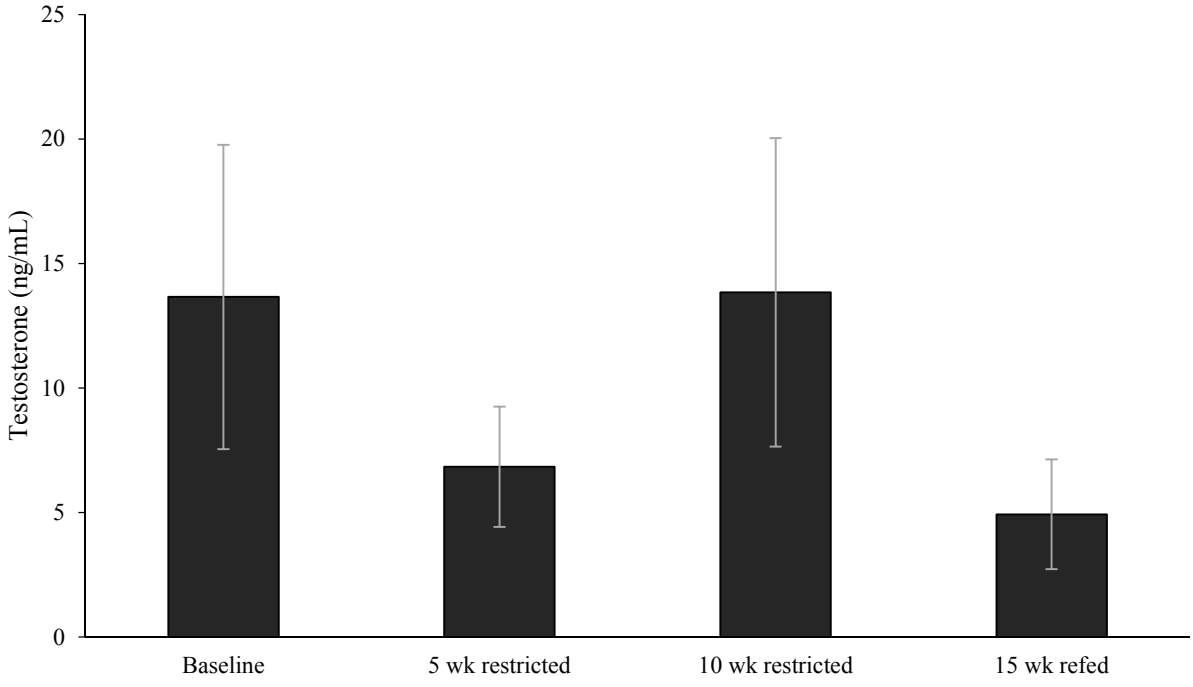

Figure 3. Mean (±SE) circulating testosterone concentrations in male *Lamprophis fuliginosus* (n = 5, 8, 4, 4).

Innate immune measures

Bacterial killing capacity

There was a significant effect of food restriction and refeeding (RM ANOVA $F_{3, 48}$= 6.71, p = 0.001) on bacterial killing capacity, but no effect of sex ($F_{1, 16}$= 0.39, p = 0.54) or interaction between food restriction and sex ($F_{3, 48}$= 2.13, p = 0.11, Fig. 4). The mean bacterial killing capacity was 46.5 ± 42.0%. Sidak corrected pairwise comparisons indicated that there was a significant difference between week 5 and week 10 (p = 0.04), baseline and week 15 (p = 0.02) and week 10 and week 15 (p = .03). The difference between baseline and week 5 bacterial killing capacity approached significance (p = 0.06).

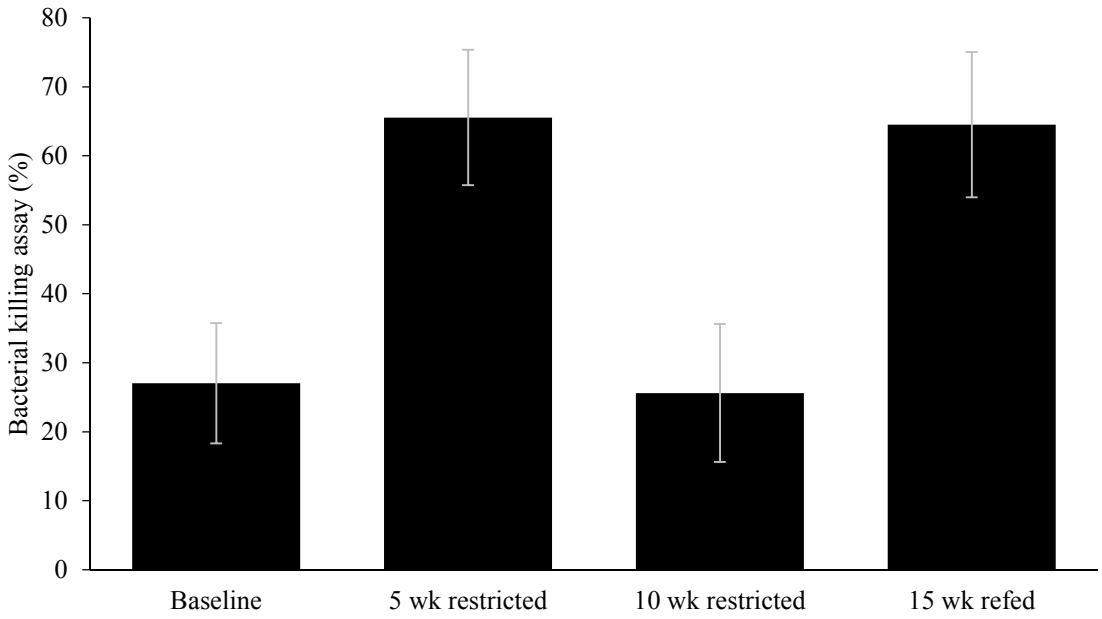

Figure 4. Mean (±SE) circulating bacterial killing capacity in male and female *Lamprophis fuliginosus*. Snakes were fed 5% of their body mass every two weeks during the 10 week period of food restriction. After the 10 week sampling period, snakes were fed *ad lib* every week for the final 5 weeks of the study (n = 24, 24, 24, 19).

Hemolysis titers

There was no effect of food restriction or refeeding ($x^2 = 6.759$, $p = 0.08$) or an effect of sex on the lytic ability of snake plasma ($x^2 = 8.542$, $p = 0.287$, Fig. 5).

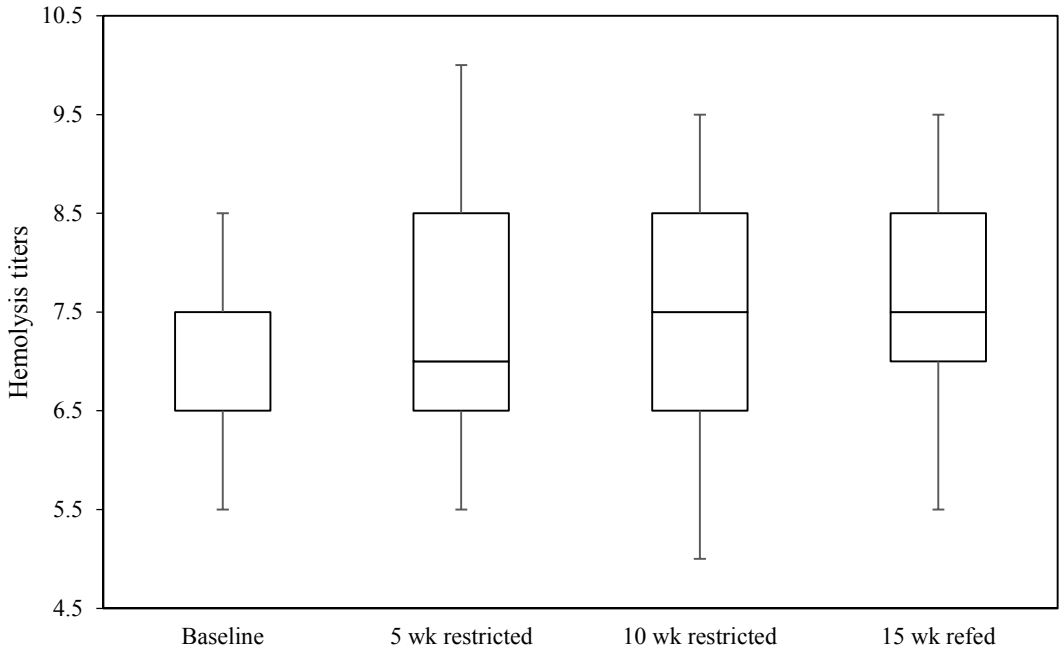

Figure 5. Hemolysis titers during each time period of the 15 week study in female and male *Lamprophis fuliginosus*. The box plots represents the median, upper and lower quartiles, and the range of values. (n = 24, 24, 24, 22). No median is shown for baseline due to identical values for the first quartile and median.

White blood cell counts

The number of WBCs per 10,000 RBCs was significantly lower in snakes when they were food restricted for 10 weeks than at the start of the study (paired $t = 3.72$, $p = 0.007$, Fig.6). Ten weeks of food restriction did not, however, affect the mean number of heterophils per 10,000 RBCs (paired $t = -0.04$, $p = 0.97$). But the heterophil to lymphocyte ratio (H:L) was significantly greater when snakes were food restricted for 10 weeks than at the start of the study (paired $t = -3.20$, $p = 0.02$, Fig. 7).

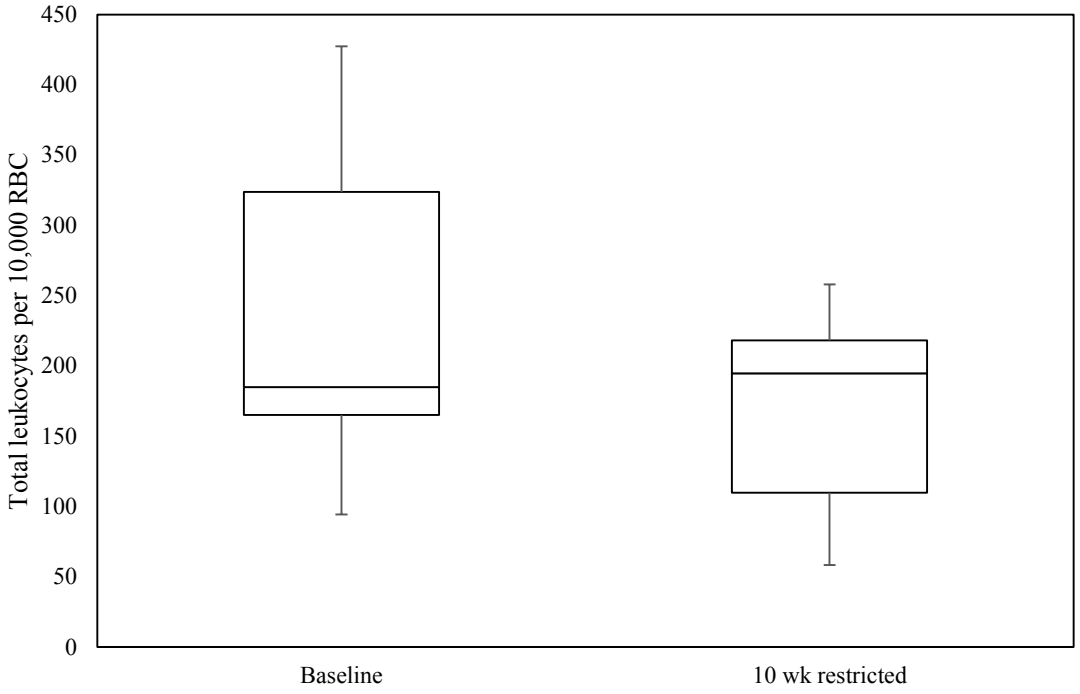

Figure 6. Total leukocytes per 10,000 RBC in female and male *Lamprophis fuliginosus*. Eight animals were used for statistical analysis (female, n = 4 and male, n = 4). The box plot represents the median, upper and lower quartiles, and the range of values.

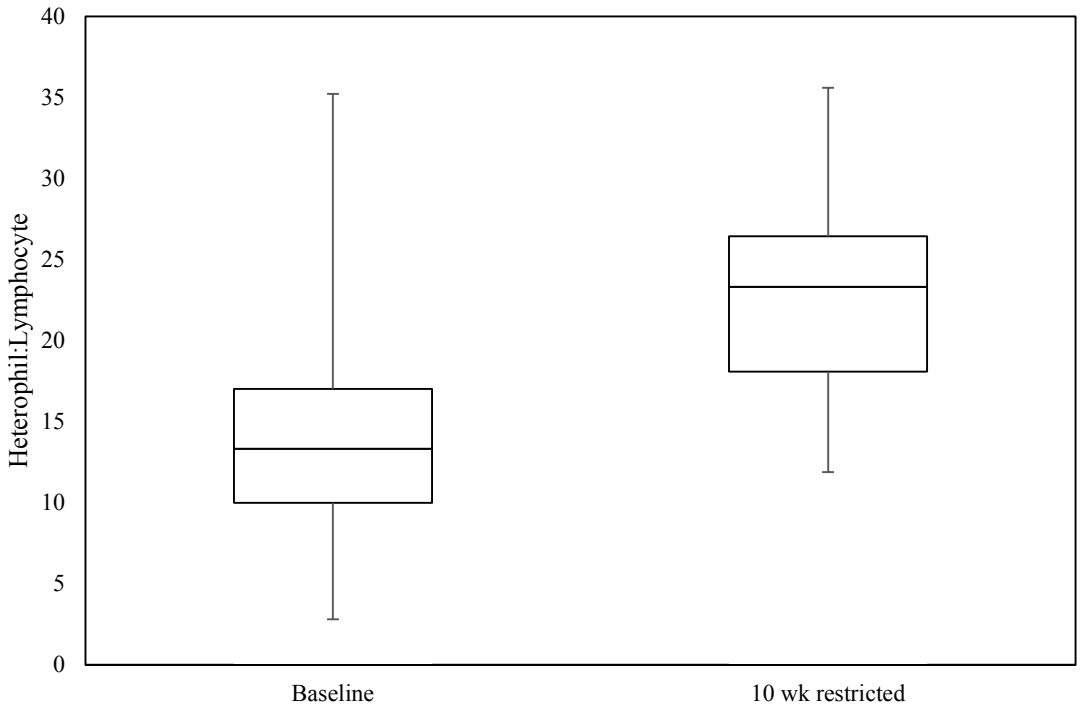

Figure 7. The heterophil to lymphocyte ratio (H:L) in female and male *Lamprophis fuliginosus*. Eight animals were used for statistical analysis (female, n = 4 and male, n = 4). The box plots represents the median, upper and lower quartiles, and the range of values.

Triglyceride

There was not an effect of food restriction or refeeding ($x^2 = 3.13$, $p = 0.37$) or an effect of sex ($x^2 = 5.58$, $p = 0.59$) on plasma triglyceride (Fig. 8). The mean plasma triglyceride concentration for females was 117.8 ± 41.2 mg/dL; whereas, the mean for males was 23.8 ± 6.8 mg/dL.

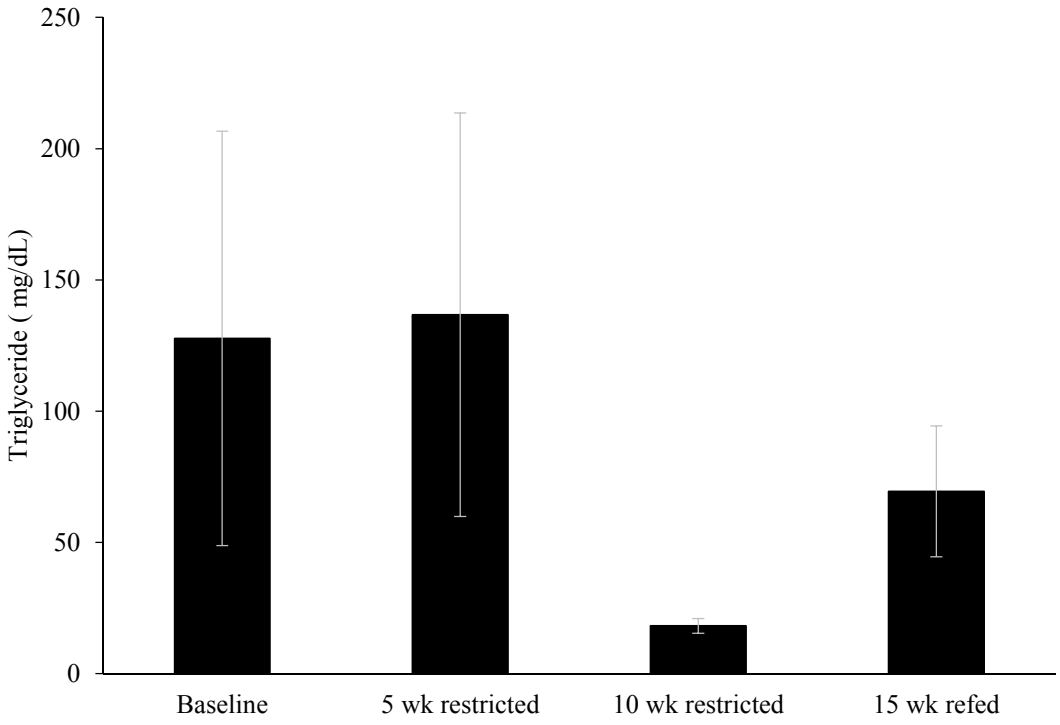

Figure 8. Mean (±SE) circulating triglyceride concentrations in female and male *Lamprophis fuliginosus.* Standard errors shown for each time period. (n = 23, 24, 23, 21).

Uric acid

There was no significant effect of food restriction ($x^2 = 4.86$, $p = 0.18$) or an effect of sex ($x^2 = 1.36$, $p = 0.98$) on plasma levels of uric acid (Fig. 9). The mean plasma uric acid concentration for females was 6.4 ± 0.4 mg/dL; whereas, the mean for males was 6.4 ± 0.4 mg/dL.

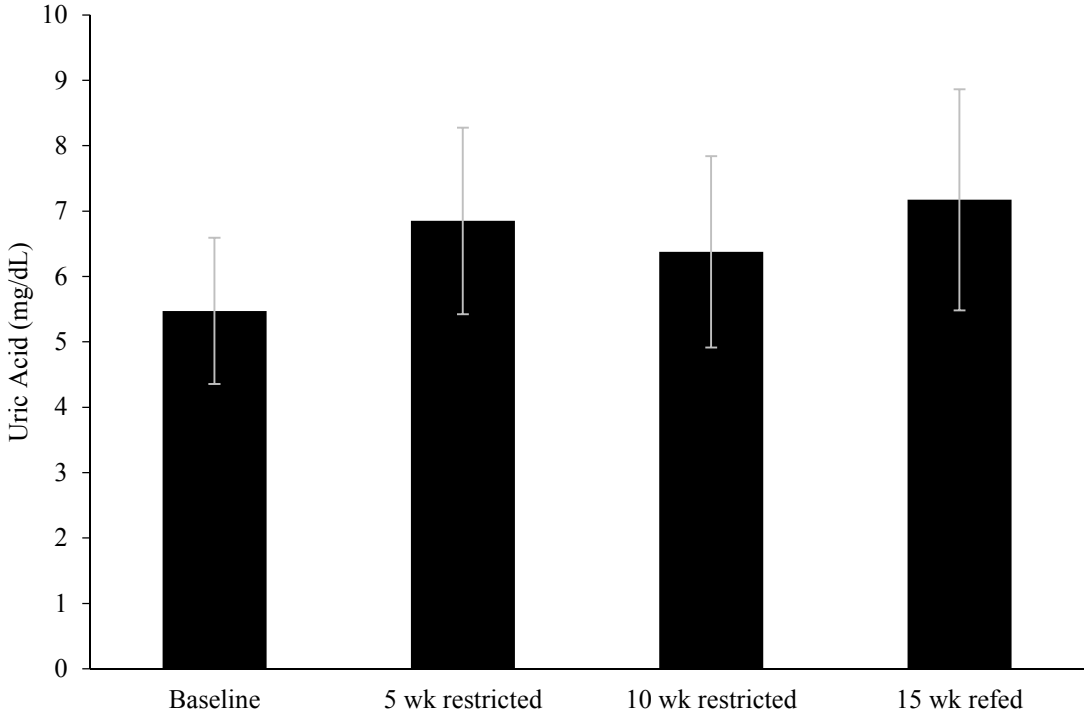

Figure 9. Mean (±SE) circulating uric acid concentrations in female and male *Lamprophis fuliginosus.* (n = 20, 23, 19, 18).

Associations involving percent body mass loss over the 10 weeks of food restriction

Plasma corticosterone concentration after 10 weeks of food restriction was significantly positively correlated with the percent body mass loss (Table 2); however, this relationship was not significant after exclusion of an outlier (CORT 4,000 ng/mL). The percent mass loss over the 10 weeks of food restriction was also not significantly associated with any of the innate immunity measures (Table 2).

Table 2.

Correlations between percent body mass loss after 10 weeks of food restriction and corticosterone, corticosterone without outlier, bacterial killing capacity, lysis titers, and white blood cell counts

	Spearman R_s	*N*	*P*
Corticosterone (ng/ml)	0.62	24	0.001
Corticosterone(ng/ml)*	0.247	23	0.256
Bacterial killing capacity (%)	-0.04	23	0.88
Hemolysis titer	0.12	24	0.58
Total leukocytes/10,000 rbc	-0.08	16	0.78
Heterophils/10,000 rbc	-0.09	16	0.72
Heterophils:Lymphocytes	-0.09	16	0.74

CHAPTER IV

DISCUSSION

The purpose of this study was to document the effect of food restriction on plasma CORT, innate immunity, and blood chemistry in *Lamprophis fuliginosus*. During the period of food restriction, food availability was reduced from 20% of body mass twice a week to just 5% of body mass once every two weeks. Body mass was significantly reduced by 10 weeks of food restriction. There was also a significant increase in body mass after 5 weeks of *ad lib* feeding. Males exhibited a greater response to food restriction than females as evidenced by the significant interaction between food restriction and sex. With the sexually dimorphic nature of this species, males may be more prone to rapid weight loss.

Although food restriction has been shown to significantly elevate corticosterone in snakes (Neuman-Lee *et al*., 2015), CORT was not affected by 10 weeks of food restriction in *Lamprophis fuliginosus*, in spite of the reduction in body mass. The percent body mass loss was positively correlated with CORT concentrations, which may indicate a relationship, but this significance was driven by one male with especially high CORT levels that lost the largest percent in body mass and subsequently died. Presumably, for this species, a more intense food restriction (e.g., complete deprivation) is required to elicit a significant CORT response. The lack of sensitivity to food restriction may be either specific to the life history of this species, or it represents a broader occurrence existing within reptiles. Insensitivity of the HPA axis, the time of blood sampling, and intrinsic variation with the species could also affect CORT concentrations found in this

study. *Lamprophis fuliginosus* is a nocturnal species with peak activity during the scotophase of the photoperiod (Lutterschmidt *et al*., 2002). Peak activity for this species is between 1900 hr and 2300 hr. Because of this nocturnal pattern, CORT presumably exhibits a diel rhythm. Glucocorticoid diel rhythms have been reported in many vertebrates. For example, in birds such as the Nazca booby (*Sulu granti*) there is a clear diel CORT cycle in which CORT peaks at the beginning of the activity period (Tarlow *et al*., 2003). This peak in CORT is assumed to prepare the body for the energy requirements associated with increased physical activity (Tarlow *et al*., 2003). In reptiles, this diel peak in CORT has been reported in *Dipsosaurus dorsalis* (Callard and Chan, 1972), *Lacerta vivipara* (Dauphin-Villemant and Xavier, 1987), *Chelonia mydas* (Jessop *et al*., 2002), and *Egernia whitii* (Jones and Bell, 2004). If blood samples are taken without regard to this peak time period, the CORT values could be biased (Tarlow *et al*., 2003). For this study, blood sampling began at 900 hr and concluded at 1500 hr, which would have been before the active period of this species and subsequently before the proposed CORT peak. This blood sampling protocol would therefore have most likely captured the basal concentrations of CORT.

In *Lamprophis fuliginosus* it has been shown that food availability exhibits an effect on life history traits (i.e., age of maturity and age at first reproduction): on a high diet regime, this species can reproduce within seven months (Byars *et al*., 2010). However as with CORT, there was no significant effect of food restriction on plasma concentrations of estradiol in females or testosterone in males. There was a tendency for a reduction in plasma estradiol after 10 weeks of food restriction, but surprisingly no increase with *ad lib* feeding. Food manipulation did not have a clear effect on plasma

testosterone, perhaps in part because of the small sample size. Plasma testosterone had a tendency to decrease after 5 weeks of food restriction and then increase after 10 weeks, a change that is difficult to explain. Surprisingly, *ad lib* feeding seems to have decreased plasma testosterone.

Although useful as an indicator of stress, changes in CORT alone do not fully characterize the stress response (Dhabhar, 2002; Prince *et al*., 2013; Neuman-Lee *et al*., 2015). Other physiological systems are affected by stress. This study indicated that food restriction altered multiple aspects of innate immunity. Traditionally, the relationship between CORT and the immune system is attributed to energetic investments (Dhabhar, 2002; Prince *et al*., 2013; Neuman-Lee *et al*., 2015). Immune function may be enhanced initially while energy is abundant, but then become suppressed as energy is diverted to other more important systems (Dhabhar, 2002). In this study the bacterial killing capacity of snake plasma was significantly affected by food manipulation and exhibited both enhancement and suppression: bacterial killing capacity after 5 weeks of food restriction was nearly significantly higher than baseline capacity, but bacterial killing capacity after 10 weeks of food restriction was significantly lower than both 5 week and 15 week values. Interestingly, after 5 weeks of *ab lib* feeding, bacterial killing capacity was significantly greater than baseline values. This enhancement may be related to energy availability (Moore and Jessop, 2003; Dhabhar, 2009; Neuman-Lee *et al*., 2015). Although nonsignificant, this tendency of enhancement was also observed in the lytic ability of snake plasma: baseline lytic capability was lower than those observed after food restriction and the *ad lib* feeding period. As with bacterial killing capacity at 10 weeks, immunosuppression was also observed in leukocyte concentrations: total leukocytes per

10,000 RBC were significantly lower after 10 weeks of food restriction. The data suggest a relationship between total leukocytes counts and the heterophil to lymphocyte ratio: as total leukocytes decreased the ratio of heterophil to lymphocytes increased significantly. This increase in circulating heterophils could constitute a redistribution of immune cells. This redistribution does not reflect a loss of cells but a reorganization to specific tissues. Cells have been shown to be transported from the blood stream to organs (i.e. lymph nodes, skin) (Dhabhar, 2002). This redistribution is proposed to be an adaptive mechanism to increase leukocyte concentrations in key organs (Dhabhar *et al*., 1994). The increase observed in heterophils to lymphocytes could be attributed to the transportation of lymphocytes from blood to the skin (Dhabhar *et al*., 2000; Dhabhar, 2002). In fasting Herring Gulls, *Larus argentatus*, redistribution has been observed after 6 days: blood concentrations of lymphocytes decreased while heterophils increased significantly (Totzke *et al*., 1999).

The use of plasma concentrations of triglyceride and uric acid illustrate the effect of food restriction stress on metabolic state (Price *et al*., 2013). After 10 weeks of food restriction, triglyceride concentrations were observably lower than those at baseline. This observed decrease suggests that the snakes were energetically stressed in spite of unchanged CORT levels. After feeding, macronutrients are absorbed and transported to the liver to be converted into triglyceride (Price *et al*., 2013; Neuman-Lee *et al*., 2015). Triglycerides are found at the highest levels in the blood stream after feeding as the compound is transported for storage in the adipose tissue (Christel and DeNardo, 2007; Price *et al*., 2013). These levels can also stay elevated for several days after feeding depending on the study species (Christel and DeNardo, 2007; Price *et al*., 2013). This

could explain the observed increase of plasma triglyceride concentrations after 5 weeks of the *ad lib* feeding period.

Uric acid concentrations are highly context dependent and should be considered when interpreting metabolic status (Totzke *et al*., 1999). Uric acid is associated with protein catabolism and has been documented to increase during long fasting periods and digestion of food sources (Cherel *et al*., 1988; Boismenu *et al*., 1992; Totzke *et al*., 1999). The elevation of plasma uric acid levels observed during fasting is typically associated with severe food restriction and muscle degradation (Cherel *et al*., 1988). In the King Penguin, *Aptenodytes patagonicus*, no significant increase in uric acid occurs until after 30-40 days of fasting (Cherel *et al*., 1988). The current study found no significant effect of 10 weeks food restriction on plasma uric acid in African brown house snakes, suggesting that 10 weeks of restricted feedings was not an especially severe restriction for *L. fuliginosus*. Uric acid can also increase after feedings as proteins are digested (Maixner *et al*., 1987). The observed increase in plasma uric acid concentration after 5 weeks of *ad lib* feeding could be attributed to the digestion of food sources.

Overall, the results from this study indicate that African brown house snakes are remarkably tolerant of extended periods of food restriction. Although ten weeks of food restriction resulted in a significant loss of body mass, food restriction failed to elevate plasma CORT concentrations and did not suppress either plasma testosterone in males or plasma estradiol in females. Food restriction appeared to have complex effects on innate immunity, as there was evidence of both immunoenhancement and immunosuppression in bacterial killing capacity and leukocyte counts. No differences attributed to sex were

observed in any measure other than body mass loss, which might be associated with either small sample sizes or a lack of severity of the stressor. Lastly, my prediction that percent body mass loss would be positively correlated with CORT and negatively correlated with innate immunity measures was also not supported. To further explore the effect of food restriction on hormones, immunity, and blood chemistry, future studies should subject snakes to complete food deprivation and incorporate behavioral and metabolic measurements. For example, the snakes in this study may have mitigated the effects of food restriction by conserving energy though either reductions in physical activity or metabolic rate.

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