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Energy Stores and Life-History Transitions in Red-Sided Garter Snakes (*Thamnophis sirtalis parietalis*)

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Energy Stores and Life-History Transitions in Red-Sided Garter Snakes

(*Thamnophis sirtalis parietalis*)

by

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Abstract

All organisms must prioritize investment in either reproductive or self-maintenance activities. Despite this established paradigm, our understanding of how organisms choose to prioritize certain behaviors and physiologies over others remains limited. It is likely that an organism's energy status not only influences variation in reproductive effort, but also transitions to and from reproductive activities. My dissertation aims to investigate how energy metrics (body condition index, adipocyte follicle size, and liver glycogen) relate to reproduction and associated life-history stages in red-sided garter snakes (*Thamnophis sirtalis parietalis*). The second chapter of my dissertation examines if energy metrics differ with migratory status and sex in red-sided garter snakes. We expected to find that snakes investing primarily in mating behaviors had elevated energy metrics compared to individuals that initiated migration to summer feeding grounds. However, we found that neither adipocyte follicle area nor liver glycogen differed with migratory status in red-sided garter snakes, and therefore a physiological mechanism underlying the decision to migrate remains elusive. In this chapter, we also found a sexual dimorphism in energy stores of red-sided garter snakes, with females having significantly larger adipocyte follicles and higher liver glycogen stores than males. In my third dissertation chapter, we aimed to investigate if energy stores influence reproductive behavior. We altered a snake's perception of adipose stores by injecting the peptide hormone leptin, as it is produced and secreted from adipocytes in proportion to

cell size and thus accurately indicates the amount of stored fat. While leptin increased both appetitive and consummatory sex behaviors (e.g., courtship score and number of copulations, respectively) in male snakes, we found leptin only increased consummatory sex behavior in females. These results indicate that leptin exhibited sex-specific effects on reproductive behavior in red-sided garter snakes. Because energy metrics influenced reproductive behavior in red-sided garter snakes, in my fourth dissertation chapter we aimed to determine how hibernation duration and temperature influence energy metrics. Our findings suggested that red-sided garter snakes preferentially utilized liver glycogen stores for energetic demands during hibernation, a finding that conflicts with the established literature in mammals indicating the preferential use of adipose stores. Additionally, exposure to elevated temperatures during hibernation depleted energy stores to a greater extent than an ecologically relevant hibernation temperature. Expending more energy during hibernation results in less available energy for mating activities upon spring emergence. Collectively, these data support the hypothesis that energy stores help an organism pinpoint the appropriate time to invest in certain behaviors and physiologies, which in turn will increase its Darwinian fitness. In examining multiple stages of an animal's annual life-history cycle and connecting physiology to behavior and subsequently fitness, my dissertation provides a view into how exposure to different temperatures affects a whole organism's biology.

Dedication

I dedicate this dissertation to anyone
that has ever been made to feel that fat isn't sexy.
Here, I provide scientific evidence
to suggest otherwise.

Acknowledgments

First, I would like to acknowledge everyone, ranging from students to faculty and staff that have influenced my time at Portland State. No matter how small the contribution, you have provided me with invaluable experience that has shaped me as an individual, teacher, and researcher. I could not have accomplished this dissertation without the careful consideration, feedback, and support from my advisor, Deborah Lutterschmidt. Thank you. My committee provided me with a wide perspective that I hope to carry throughout my career. Thank you Bradley Buckley, Michael LeMaster, Michael Murphy, and Daniel Taylor-Rodriguez. I would like to acknowledge my ‘men’ on the outside, Jason Podrabsky and Radhika Reddy, for their timely reassurance and assistance. I appreciate everyone in the Lutterschmidt Lab that has helped to mold the researcher I am today and has provided me with friendship and community for the past five years: Deborah Lutterschmidt, Catherine Dayger-Forbes, Ashley Lucas, Kalera Stratton, Holden Anderson, Bradley Cumez, Alonso Delgado Covarrubias, Nathan Colven, Roslyn Honodel, Francine Iopu, Christopher Lundrigan, Stephanie Martin, Lauren Merlino, Nichole Proctor, and I am particularly appreciative of Treven Winters. Daniel Zajic, thank you for so many things: baked treats, cocktails and dinner, friendship, and being the best peer mentor I didn't know I needed. And a special thank you to all my family and friends that have supported me through the years. Lastly, to my swan, Elizabeth Brandlin, I would not be the person I am today without your unwavering support and love for the past 15 years.

Table of Contents

List of Tables

Chapter 3

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Preface

Chapter 2 is in press for *Physiological and Biochemical Zoology*.

Wilson, R.C. and D.I. Lutterschmidt. 2020. Energy metrics of red-sided garter snakes (*Thamnophis sirtalis parietalis*) vary with sex but not life-history stage.

Chapter 3 is under review for *Hormones and Behavior*.

Wilson, R.C., LeMaster, M.P., Lutterschmidt, D.I. Exogenous leptin promotes reproductive behavior during aphagia in red-sided garter snakes (*Thamnophis sirtalis parietalis*).

Chapter 4 will be submitted to the *Journal of Experimental Biology*.

Chapter 1

Introduction

A common, well-established biological paradigm concerns the necessity of an organism to prioritize either reproductive or self-maintenance activities (Bonnet et al. 2002; Fletcher et al. 2013; Lourdais et al. 2013; Dupoué and Lourdais 2014). To increase fitness, an organism must both survive and reproduce. To some extent, longer survival times increase the likelihood of successful reproductive opportunities. Further, deciding when to invest in reproduction can influence an organism's probability of surviving. (Jouventin and Dobson 2002; Bohec et al. 2007). Coordinating the timing of reproduction to coincide with favorable environmental conditions not only increases the survivability of an organism, but also the survival of that organism's progeny.

Interestingly, not all individuals invest in reproduction at the exact same time during the reproductive season, and individuals exhibit variation in reproductive investment (Broderick et al. 2003). An organism receives information pertaining to the environment through multiple exogenous cues (e.g., photoperiod and temperature), which in turn affect an organism's physiology (Pinter and Negus 1965; Björnsson et al. 1989; Bartness 1996; Demas and Nelson 1998; Larsen et al. 2001; Niva and Takeda 2003; Lutterschmidt and Mason 2009; Zajac et al. 2011; Lutterschmidt 2012) and dictate the appropriate season when an organism should invest in reproduction (i.e., spring vs. fall

breeders). However, to what extent these exogenous cues affect physiology may be context dependent (Ozaki et al. 1978; San Martin and Touitou 2000; Barrett et al. 2007). Such context dependency can be contingent upon, but not limited to, resource availability and energy status (i.e., high body condition index, large fat stores, etc.). Because reproduction is an energetically expensive activity, the amount of stored energy substrates likely plays a role in pinpointing the appropriate time for an organism to reproduce.

While an organism can utilize three energy substrates in the form of glucose, fatty acids, and amino acids, only glucose and fatty acids can be stored without functional loss upon catabolism. Glucose can be stored as glycogen in the liver as a systemic reservoir, but also in metabolically active tissues such as skeletal muscles. Fatty acids are stored in the form of triglycerides in adipocyte follicles. Visceral or subcutaneous adipose tissues serve as a reservoir of triglycerides for an organism. As with glycogen, some tissues also have their own reservoirs of stored fat (e.g., muscle and liver). Amino acids, the third metabolic substrate, do not have a stored form and the catabolism of proteins can result in the functional loss of tissues such as muscles. Historically, animals are thought to primarily utilize glucose, and then resort to fatty acid use when glycogen levels are substantially depleted. Only in times of severe need, such as starvation, are proteins thought to be catabolized for fueling physiological processes (Secor and Carey 2016). However, recent studies suggest that organisms dynamically utilize different energy stores based on an animal's behavior or activity (Costanzo et al. 2013; Jenni-Eiermann 2017; Guglielmo 2018). Investigating if and when animals

preferentially utilize certain energy substrates over others will provide insight into whether energetic demands of similar activities differ across species, and also illuminate to what extent animals adhere to or depart from the historical perspective of the prioritization of energy substrates.

Life histories and energy stores

An organism will experience a variety of phases throughout its lifetime. These different phases are referred to as life-history stages and can range from activities such as birth, development, sexual maturation, reproduction, and senescence. While there are many nuances in a single life-history stage across species and even unique stages specific to certain species, all organisms must engage in reproduction for lineages to persist. A multitude of evidence across taxa suggests that energy availability influences history-life stage duration (Bronson and Marsteller 1985; De Block and Stoks 2005; Stallings et al. 2010; Muir et al. 2013; McBride et al. 2015; McCann and Padilla 2015) or strategy (Alonso-Fernández and Saborido-Rey 2012; Becker et al. 2013). Reproduction is arguably the most commonly studied life-history stage as it relates to energy availability (Bronson and Marsteller 1985; Doughty and Shine 1997, 1998; Kirk 1997; Barron and Andraso 2001; Smith and Moore 2003; Groscolas et al. 2008; Alonso-Fernández and Saborido-Rey 2012; Becker et al. 2013). Liver glycogen decreases from the breeding to the post-breeding season in female Woodhouse's toads (*Anaxyrus woodhousii*), suggesting that glycogen is utilized for reproductive activities (Long 1987). Interestingly, male toads do not show a

change in liver glycogen across seasons (Long, 1987). Male and female American Redstarts (*Setophaga ruticillia*) arriving at the breeding grounds with higher fat scores have higher average egg masses (Smith and Moore 2003). Female Southern water skinks (*Eulamprus tympanum*) producing offspring show higher losses of energy substrates that are stored in the tail, as measured by decreasing tail width (Doughty and Shine 1998). Female red-sided garter snakes (*Thamnophis sirtalis parietalis*) with a positive body condition index mate more quickly than females in negative body condition (Dayger et al. 2013), and female water snakes (*Nerodia sipedon*) fed a high caloric diet develop more ovarian follicles compared to conspecifics fed a low caloric diet (Barron and Andraso 2001). Further, food deprivation of female rats can delay the reproductive activities (Bronson and Marsteller 1985), and insufficient energy stores can result in premature ending of reproduction for king penguins (Groscolas et al. 2008). Collectively, these lines of evidence suggest a crucial role of energy status in determining when and how much an individual invests in reproduction.

The majority of the aforementioned studies utilized proxies (i.e., body condition index, tail width, etc.) to relate energy status to reproduction. Studies directly measuring the relationship between energy substrates and reproduction are limited (but see Barros et al., 2013; Box et al., 2010) and mainly focus on one energy substrate (i.e., fatty acid stores or liver glycogen). Although these studies are meaningful in furthering our understanding of the energy requirements of reproduction, we are unable to determine if one energy substrate is utilized over

another. Lastly, to what extent energy stores influence transitions to and from reproductive life-history stages has received far less consideration.

Appropriating a signal that reflects stored energy status

As discussed above, numerous observational studies suggest that the amount of stored energy positively correlates with reproduction. Causal evidence to support the assumption that stored energy substrates influence reproduction is often accomplished through dietary and caloric manipulations (Gregory and Skebo 1998; Barron and Andraso 2001; Sirotkin et al. 2008). However, altering an organism's diet and/or caloric intake does not necessarily affect stored energy substrates without corroborating studies, which are often absent. Methods that alter diet or caloric intake also lack the ability to discern which stored energy substrate (i.e., fatty acids or glycogen) is primarily utilized for reproduction.

Direct manipulations altering the amount of stored energy would provide causal evidence to connect organismal energy status and reproduction. In addition to acting as a reservoir for fatty acids, adipocytes produce and secrete the peptide hormone leptin in proportion to cell size. Leptin accurately signals energy reserves in numerous species including domestic livestock, mice, and Arctic foxes (Frederich et al. 1995; Delavaud et al. 2000; Fuglei et al. 2004). Hence, I will administer leptin exogenously as a proxy to alter an organism's perceived size of adipocytes and therefore the amount of stored fatty acids. Because the research that investigates how leptin influences reproduction focuses almost exclusively on mammalian models, investigations into how leptin

affects reproduction in a nontraditional model will provide a comparative viewpoint as to whether the role of leptin is conserved across taxa. In mammals and one species of lizard studied to date, leptin stimulates reproductive behavior and physiology (Wade et al. 1997; Finn et al. 1998; Schneider et al. 2007; Putti et al. 2009). However, leptin inhibits reproductive behavior in food-deprived Syrian hamsters (Wade et al. 1997). Therefore, investigating if leptin promotes or inhibits reproductive behavior in an organism experiencing predictable bouts of aphagia, such as northern populations of red-sided garter snakes will help to determine if the role of leptin is constrained by ecological/environmental conditions or phylogeny.

Energy metrics and life-history stages surrounding reproduction

For many organisms, reproduction is both temporally and geographically separated from other life-history stages. Such organisms must migrate, sometimes upwards of thousands of kilometers to transition between reproduction and other life-history stages. For long-distance migrations, birds mainly utilize fat, but also catabolize proteins to produce metabolic water and prevent dehydration (Butler 2016; Jenni-Eiermann 2017). Unlike fat and glucose, no stored form of proteins exists, and catabolizing substantial amounts of proteins can cause the functional loss of protein-rich tissues such as muscles. As such, the majority of migrating birds primarily utilize fat stores and only utilize proteins for approximately 5% of their metabolic budget to ensure the structural integrity of flight muscles (Jenni-Eiermann 2017). Although research investigating

which energy stores fuel long-distance migrations in bats is in its infancy, evidence suggests that bats utilize a mixed energy substrate approach with ingested nutrients acquired during migration and fat stores to fuel energy demands for long distance flights (Guglielmo 2018). Surprisingly, research into the relationship between migration and energy stores in large bodied mammals is sparse. However, the expression of lipolytic genes in the blubber of migrating Bowhead whales (*Balaena mysticetus*) is significantly higher in the spring prior to arrival at summer feeding grounds compared to individuals migrating away from summer feeding grounds in the fall (Ball et al. 2017). This study only investigated adipose metrics preventing the drawing of any conclusions regarding the prioritization of energy substrates to fuel energy demands during migration in whales.

Sea turtles and fishes are two ectotherms that represent long-distance migratory swimmers. Although research into migrations of sea turtles is robust, research examining other reptilian migrators is sparse. Sea turtles also likely utilize adipose stores to fuel energetic demands during migration as plasma triglycerides are elevated prior to migration compared to Green sea turtles (*Chelonia mydas*) arrived at their breeding grounds (Southwood and Avens 2010). Investigations into migratory behavior and physiology of fishes have been robust, especially in those species that have commercial and economic value such as anadromous fishes. Sockeye salmon (*Oncorhynchus nerka*) migrating longer distances with higher elevation gains have elevated lipid stores and fewer eggs than conspecifics migrating shorter distances with less gains in elevation

(Crossin et al. 2004). Muscle tissue of Chinook salmon (*Oncorhynchus tshawytscha*) sampled early in their migration had higher lipid content than individuals sampled later (Mesa and Magie 2006). American shad (*Alosa sapidissima*) primarily utilize lipids during migration, but also utilize proteins to a lesser extent (Leonard and McCormick 1999). In their migrations to and from breeding groups in the ocean, male white-streaked groupers (*Epinephelus ongus*) with higher lipid stores spent more time at breeding grounds compared to resident areas (Kawabata et al. 2015).

Research into amphibian migrations has received little attention particularly as it pertains to energy stores. This area of research is sparse likely due to several difficulties in studying amphibian movements. Because many amphibian populations exist as metapopulations, it can be difficult to classify movements as migrations because organisms can visit multiple sites within a metapopulation prior to returning to their initial patch (Semlitsch 2008). Although historically only bidirectional movements were classified as migrations, some unidirectional movements are now classified as migrations (Southwood and Avens 2010). For instance, an organism may perform several unidirectional movements between metapopulations prior to returning to its original location this can be classified as migration, although this idea is controversial. It is also difficult to sample migratory and non-migratory amphibians due to the infrequent and solitary nature of migrations reaching upwards of 1 km (Semlitsch 2008; Sinsch 2014). Further, lethal methods that are commonly used to quantify specific energy stores are often unrealistic due to global population declines of

amphibians. However, the characterization of some amphibian populations as partial migrators can provide insights into the energetic cost of amphibian migrations.

Partial migration of populations entails a subset of a population migrating away from breeding ponds, while other individuals reside at the pond until the next breeding season (i.e., residents). Migratory male red-spotted newts (*Notophthalmus viridescens viridenscens*) took longer to grow tail fins to the appropriate height necessary to compete for mating opportunities compared to resident males, which suggests that energy stores of migratory males is likely lower than residents (Bloch and Grayson 2010). Further, preliminary data suggests that the body condition of migrant red-spotted newts is lower than residents at the end of the breeding season (Grayson et al. 2011). In a study on alpine newts (*Icthyosaura alpestris*) and smooth newts (*Lissotriton vulgaris*), body condition index relates to migratory status in a species- and sex-specific manner: only female alpine newts that left the breeding pond displayed a significant decrease in body condition index relative to resident conspecifics (Mettouris et al. 2018). The relationship between body condition and number of visits to multiple breeding ponds in another species of alpine newt (*Mesotriton alpestris*) differed depending on the population examined (Kopecký et al., 2010). Because fat stores significantly correlated with body condition index in newts (Denoël et al. 2002), these studies suggest a relationship between amphibian migrations and energy status. However, this context dependency of sex and population suggests that other factors (e.g., population density and

environmental factors) contribute to the decision to migrate in amphibians. Further, the relative short-distance of migrations (i.e., upwards of 1 km in amphibians versus thousands of kilometers in bats and birds) with frequent feeding opportunities may point to a less critical role of energy status in amphibian migrations compared to other taxa. However, investigations into long-distance migrations (i.e., 15 km) such as those exhibited by Pool frogs (*Pelophylax lessonae*; Sinsch, 2014) must be conducted to determine if energy status relates to migration in amphibians.

Regardless, this evidence across a wide range of vertebrate taxa points to energy stores, particularly energy stored as fat, as contributing significantly to fueling the energetic demands of migration. However, numerous clades of animals are missing from such investigations such as frogs, lizards, and snakes. Of these three taxa, snakes generally migrate longer distances of approximately 10 km as compared to 1-3 km for frogs and lizards (Semlitsch 2008; Southwood and Avens 2010; Sinsch 2014). With distances of approximately 10 km, snakes may not be classified as long-distance migrators as compared to some species capable of migrations ranging from hundreds to thousands of kilometers (e.g., birds, bats, and sea turtles). However, considering migratory distance as the sole metric in determining migratory difficulty may be misleading when examining the total effort needed to accomplish migrations. Differing modes of locomotion are more costly than others (Walton et al. 1990; Butler 2016). The form of slithering (i.e., lateral undulation) utilized by many snake species including one of the longest terrestrial migrators in reptiles, the red-sided garter snakes, is as costly

as running (Walton et al. 1990; Butler 2016). Running is the most costly form of locomotion, while swimming is the cheapest and flying intermediate to swimming and running (Walton et al. 1990; Butler 2016). The directionality and strength of currents should also be considered to obtain a more accurate view of migratory effort in migrating swimmers and flyers. Additionally, ectotherms have some innate physiological limitations that constrict metabolic rate such as lower enzymatic activity and oxygen consumption in the mitochondria, smaller surface area for gas exchange in the lungs, and a three-chambered heart (Southwood and Avens 2010). These likely augment the difficulty of shorter migratory distances in reptiles. Therefore, further investigations into reptilian migration could provide insight into how energy stores influence not only migratory distance, but also migratory effort. Expanding investigations of migration to include under-studied taxa could help clarify the ecological influences and physiological constraints that dictate energy stores not only impact migration, but also subsequent transitions to other life-history stages such as reproduction.

Another life-history stage that often occurs in proximity to the reproductive season is hibernation. By far, the majority of literature on how energy stores influence hibernation focuses on mammals (Farley and Robbins 1995; Humphries et al. 2003; Bozinovic et al. 2007; Costanzo et al. 2013; Muir et al. 2013; Bieber et al. 2014; Pigeon et al. 2016). The body mass lost by black and grizzly bears (*Ursus americanus* and *Ursus arctos horribilis*) during hibernation relates almost exclusively to lipid use (Farley and Robbins 1995). Additionally, edible dormice (G*lis glis*) with larger fat reserves display shorter bouts of torpor, a

stage characterized by decreased body temperature (Bieber et al. 2014). Individuals benefit from shorter bouts of torpor because longer torpor results in increased in water loss, an accumulation of metabolic wastes, and lower immune function (Willis 2017). Conversely, ectotherms maintain body temperatures near ambient temperature throughout winter dormancy and have different energetic demands during hibernation compared to mammals[1]. In an Alaskan population of wood frogs (*Rana sylvatica*), hibernation causes a decrease in fat body mass and muscle size, but an increase in plasma glucose levels (Costanzo et al. 2013). In juvenile tegu lizards (*Tupinambis merianae*), lipids in fat bodies and liver glycogen are significantly higher in autumn as compared to winter dormancy (Souza et al. 2004). Although adipose-metrics were not measured in a study on painted turtles (*Chrysemys picta),* liver masses of hibernating individuals were significantly lower compared to turtles sampled prior to hibernation (Muir et al. 2013). As in mammals, ectotherms utilize adipose tissue to fuel energetic costs during winter dormancy, but evidence suggests that ectotherms can also utilize liver glycogen stores for energy needs during dormancy. Expanding our knowledge through researching a wider range of taxa would not only clarify if reptiles preferentially utilize energy substrates, but could also provide insight into the differences in energetic costs of hibernation between endo- and ectothermic animals.

[1] Although winter dormancy is more accurately referred to as brumation in ectotherms, I use the term hibernation throughout this dissertation for readability and clarity.

Model organism and rationale for chapters

Incorporating novel taxa into investigations of energy substrate use during hibernation will help identify congruous changes in physiology related to this life-history stage. For instance, the increase of plasma glucose in wood frogs may be a unique adaptation allowing them to tolerate freezing body tissues (Costanzo et al. 2013). One trait that is shared by many hibernators across multiple taxa is the substantial decrease or even lapse of feeding behavior during this life-history stage. Red-sided garter snakes are not only aphagic during their winter dormancy, but also through their succeeding mating season until these snakes migrate to their summer feeding grounds located upwards of 17 km away (Gregory and Stewart 1975; Gregory 1977). While numerous organisms engage in either migration or hibernation prior to or after reproduction, red-sided garter snakes are among the few species whose mating season is preceded by hibernation and succeeded by migration. Additionally, because red-sided garter snakes exhibit aphagia prior to and through their spring mating season, they must rely solely on stored forms of energy to fuel physiological processes during multiple life-history stages. This allows the determination of how stored energy influences the behavior of an organism without the need to account for nutrients acquired during intermittent periods of feeding. Therefore, red-sided garter snakes represent a unique system to investigate how energy stores influence various life-history stages and transitions: winter dormancy, the spring mating season, and the ‘decision’ to migrate (Figure 1.1). My dissertation aims to investigate how stored forms of energy influence the annual life-history cycle of

red-sided garter snakes and will help determine how energy substrate prioritization in an ectotherm fluctuates among these life-history stages as compared to other species.

Various nuances of the annual life-history cycle of red-sided garter snakes make this system valuable in examining how energy stores influence transitions between reproduction and other life-history stages while also allowing the extension of our findings to other species. First, the annual life-history stages exhibited by one of the most northern populations of red-sided garter snakes allow for easy identification of individuals that are and are not primarily investing in mating behaviors during the spring. Upon emergence from overwintering sites, individuals from Manitoba, Canada enter an intense and short mating period all while being aphagic (Figure 1.1; Gregory and Stewart, 1975). The time spent at the den is sex specific with males spending up to two weeks courting and attempting to mate with females, whereas female snakes migrate away from the den within 24 hours of emergence (Shine et al. 2001; Lutterschmidt and Mason 2009). As mating activities decrease, snakes migrate up to 17 km to summer feeding grounds (Gregory and Stewart 1975; Gregory 1977). As this population of red-sided garter snakes exhibits a stark behavioral transition from mating to feeding activities through the initiation of migration, we can easily identify if individuals are primarily investing in reproduction or if they have transitioned to self-maintenance activities. For these reasons, I sought to determine if energy metrics influence when a snake decides to migrate in red-sided garter snakes. I predict that non-migrating snakes at the den have larger adipocyte follicles and

higher liver glycogen stores compared to migrating snakes. Further, because red-sided garter snakes exhibit a sex difference in the timing of their transition to feeding and foraging, I expect to find a corresponding sexual dimorphism in energy stores.

Prior to migration, the aphagic breeding season of red-sided garter snakes requires these snakes to rely solely on stored forms of energy to fuel intense mating activities during the spring. With the positive correlation between adipose stores and reproductive output in many other species, I aim to provide causal evidence to relate adipose tissue to reproduction by experimentally manipulating the perceived amount of stored fatty acids an animal has available for mating. To accomplish this, I utilized the hormone leptin because it is secreted from adipocytes in proportion to cell size and acts as an accurate signal of stored fat reserves (Frederich et al. 1995; Delavaud et al. 2000; Fuglei et al. 2004). In various taxa, exogenous leptin increases reproductive behavior and physiology (Wade et al. 1997; Finn et al. 1998; Schneider et al. 2007; Putti et al. 2009), but leptin inhibits reproductive behavior in food-deprived Syrian hamsters (Wade et al. 1997). Investigating how leptin influences reproductive behavior in an animal experiencing a long-term bout of aphagia allows us to determine how leptin may affect behavior in the absence of food deprivation that may cause physiological changes associated with starvation. Further, using leptin as a mimic will allow me to determine the relationship between adipocyte follicle size and mating behavior in both female and male red-sided garter snakes, an endeavor that has not been explored. Because of the predictability of aphagic bouts in red-sided garter

snakes, I predict that leptin treatment will increase mating behaviors in both male and female snakes.

Leading up to the spring mating season, red-sided garter snakes overwinter for 8-9 months in underground hibernacula and are aphagic from the onset of hibernation in fall until the end of the mating season in mid to late May. Therefore, these snakes must rely on stored fatty acids and/or carbohydrates to fuel physiological processes during and after hibernation. For my last data chapter, I investigated how energy metrics change leading up to the spring mating season. Although fatty acids are utilized primarily during hibernation in various species (Farley and Robbins 1995; Bao et al. 2010; Costanzo et al. 2013), some evidence suggests liver glycogen is the energy substrate used to fuel hibernation in garter snakes (Aleksiuk and Stewart 1971; Costanzo 1985). Eastern garter snakes (*Thamnophis sirtalis sirtalis*) emerging from simulated winter dormancy had lower levels of liver glycogen but similar fat stores compared to individuals about to enter hibernation (Costanzo 1985). Similar to eastern garter snakes, liver weights were lower in red-sided garter snakes emerging from hibernation compared to snakes entering hibernation (Aleksiuk and Stewart 1971). However, red-sided garter snakes may also utilize fat stores to fuel energy demands during hibernation, as total lipids are lower at the time of emergence compared to individuals sampled prior to hibernation. By examining energy metrics surrounding hibernation, these studies infer that energy stored in the liver and possibly fat are utilized to fuel physiological processes during hibernation in garter snakes. Direct examination of how these energy metrics

change during hibernation would unequivocally determine to what extent liver glycogen and fat stores are utilized during this life-history stage. I expect to find that snakes sampled just prior to the onset of hibernation will have the largest adipocyte follicles and highest glycogen stores in the liver, but expect to see larger decreases in liver glycogen but stable adipose stores during this life-history stage.

Significance

Together, these chapters aim to determine how red-sided garter snakes utilize energy stores over the course of their annual life-history cycle. By examining how energy metrics differ among life-history stages and to what extent they influence an individual's investment in reproduction, I aim to reveal a physiological mechanism that may inform the prioritization of certain behaviors over others. Revealing such a mechanism may inform management or ecological practices by helping to predict when an organism engages in reproductive over self-maintenance behaviors. This research may also elucidate how organisms utilize endogenous signals to coordinate physiology and behavior with optimal environmental conditions. Further, investigating the energy metrics of an organism relying solely on stored forms of energy through the majority of its annual life-history cycle will provide insight into the energetics of the vast majority of animals that are unable to exhibit such feats of aphagia. I aim to investigate how multiple forms of energy stores and energy-related factors relate to life-history stages surrounding reproduction in an ectotherm, an area of research that

is sorely missing from the literature seeking to determine how energy availability influences animal behavior.

Figures

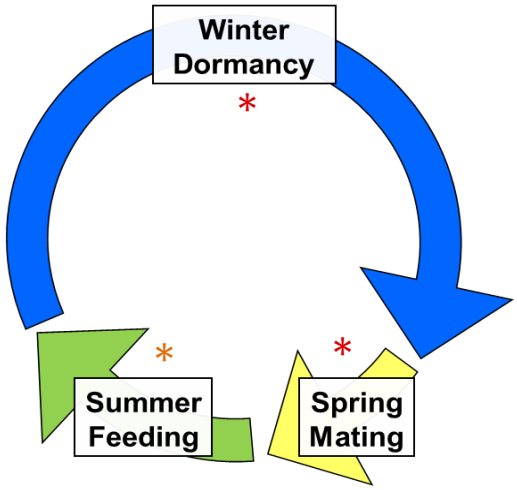

Figure 1.1. Life-history stages of red-sided garter snakes (*Thamnophis sirtalis parietalis*). Red asterisks indicate times of aphagia, while the orange asterisk indicates parturition. Modified from Krohmer and Lutterschmidt (2011).

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Chapter 2

Energy metrics of red-sided garter snakes (*Thamnophis sirtalis parietalis*) vary with sex but not life-history stage

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Abstract

Because reproduction is energetically expensive, an organism's energy stores are likely involved in mediating transitions between reproductive and self-maintenance activities. We investigated if body condition index, adipocyte follicle size, and liver glycogen differ with the life-history transition from reproduction to migration and foraging in red-sided garter snakes (*Thamnophis sirtalis parietalis*). Females primarily investing in mating behavior located at the den had a significantly higher body condition index than females migrating to summer feeding grounds. The body condition index of male snakes did not differ between those snakes located at the den or those migrating to summer feeding grounds. Neither adipocyte follicle area nor liver glycogen stores differed significantly between those snakes performing mating activities at the den and those migrating to summer feeding grounds. We did find a sexual dimorphism in that female red-sided garter snakes had significantly larger adipocyte follicles and

higher liver glycogen compared to males. Our findings support the across-species phenomenon that females and males display a sexual dimorphism in stored energy substrates. Conversely, we did not provide evidence to suggest that red-sided garter snakes primarily utilize fatty acids to fuel the initiation of migration, a finding that is not consistent with other long-distance migrators such as birds. Because we did not find evidence to suggest that stored energy metrics influence the decision to migrate, a physiological mechanism inducing migration in red-sided garter snakes remains elusive.

Introduction

A common, well-established biological paradigm concerns the trade-offs associated with investment in either reproduction or self-maintenance (Bonnet et al. 2002; Fletcher et al. 2013; Lourdais et al. 2013; Dupoué and Lourdais 2014). To increase Darwinian fitness, an organism must both survive and reproduce, and deciding when the appropriate time is to invest in reproductive activities can affect survival (Jouventin and Dobson 2002; Bohec et al. 2007). Organisms exhibiting seasonal and/or biennial reproductive cycles must coordinate the timing of reproduction with appropriate environmental conditions. Multiple exogenous cues, such as photoperiod and temperature, affect physiology (Pinter and Negus 1965; Björnsson et al. 1989; Bartness 1996; Demas and Nelson 1998; Larsen et al. 2001; Niva and Takeda 2003; Lutterschmidt and Mason 2009; Zajac et al. 2011; Lutterschmidt 2012). However, to what extent these exogenous cues affect physiology may be context dependent (Ozaki et al. 1978; San Martin

and Touitou 2000; Barrett et al. 2007). Some factors that may contribute to the context dependency of exogenous cues include resource availability and energy status of an organism (i.e., high body condition index, large fat stores, etc.). As reproduction is an energetically expensive activity, endogenous signals that reflect energy status likely influence an organism's sensitivity to exogenous signals, and perhaps facilitate transitions between life-history stages.

A multitude of evidence across taxa suggests that energy availability relates to life histories (Bronson and Marsteller 1985; Farley and Robbins 1995; De Block and Stoks 2005; Stallings et al. 2010; Alonso-Fernández and Saborido-Rey 2012; Becker et al. 2013; Costanzo et al. 2013; Muir et al. 2013; McBride et al. 2015; McCann and Padilla 2015). Reproduction has arguably been the most commonly studied life-history stage as it relates to energy availability, with higher energy stores associated with higher investment in reproduction (Bronson and Marsteller 1985; Doughty and Shine 1997, 1998; Kirk 1997; Barron and Andraso 2001; Smith and Moore 2003; Groscolas et al. 2008; Alonso-Fernández and Saborido-Rey 2012; Becker et al. 2013). Further, food deprivation delayed reproductive activities (Bronson and Marsteller 1985), and insufficient energy stores resulted in premature cessation of reproduction (Groscolas et al. 2008). This suggests energy status could potentially equip an organism with the ability to "decide" when to prioritize investment in either reproduction or self-maintenance activities. To date, little is known about the physiological mechanisms that allow organisms to prioritize life-history processes.

We investigated if metrics of energy balance, as measured by body condition index, adipocyte follicle size, and liver glycogen, differ with the life-history transition from reproduction to foraging in a well-studied population of red-sided garter snakes (*Thamnophis sirtalis parietalis*). The annual life-history stages exhibited by northern populations of red-sided garter snakes allow for easy identification of individuals that are and are not primarily investing in mating behaviors. In Manitoba, Canada, red-sided garter snakes overwinter in underground dens for up to eight months, and upon emergence enter into an intense and shortened spring mating season, all while being aphagic (Gregory and Stewart 1975). Individuals spend a variable amount of time performing mating behaviors at the den, and as the mating season ends, migrate up to 17 km to summer feeding grounds (Gregory and Stewart 1975; Gregory 1977). Male snakes emerge prior to females, and as single or small groups of females emerge, males immediately and intensely begin to court attractive females. Most female snakes remain at the den for less than one day prior to dispersing, while male snakes spend upwards of two weeks courting and attempting to mate with females (Shine et al. 2001; Lutterschmidt and Mason 2009). Because this population of red-sided garter snakes exhibits a distinct behavioral transition from mating to feeding activities that is necessarily linked with migration, we easily identified if individuals are primarily investing in reproduction or if they transitioned to self-maintenance activities (Cease et al. 2007).

Here we present data to determine if body condition index, adipocyte follicle area, and liver glycogen differ between red-sided garter snakes that were

primarily involved in reproductive activities compared to snakes that initiated migration to summer feeding grounds. Body condition index is used as a non-invasive method to obtain information about an organism's energy balance by examining the relationship between body mass and body length (Hayes and Shonkwiler, 2001). We also aimed to determine if direct measurements of stored energy (i.e., adipocyte follicle size and liver glycogen) differ between individuals at the den compared to those that initiated migration to summer feeding grounds. We predicted that energy metrics would be higher in snakes located at the den compared to individuals collected during migration. We expected to see a greater magnitude of change in adipose tissue compared to liver glycogen due to the preferential usage of fat stores in migrating birds (Jenni-Eiermann 2017). We also expected that males would shower larger differences in energy metrics because of the sex-specific differences in the timing of migration of red-sided garter snakes, in which males spend a greater time at the den compared to females. Finally, we also explored relationships among the energy metrics quantified in this study. Because organisms utilize fat as a fuel when glucose and glycogen levels are low, we did not expect to see a significant relationship between adipocyte follicle area and liver glycogen. Conversely, we did expect to see a significant, positive relationship between body condition index and both adipocyte follicle area and liver glycogen.

Material and methods

Red-sided garter snakes were collected from the Interlake region of Manitoba, Canada under the authority of Scientific Permit #WB18801 issued by the Manitoba Department of Sustainable Development. All procedures were approved under Portland State University's Institutional Animal Care and Use Committee protocol #42.

Experimental design

Male and female red-sided garter snakes were collected from 19-22 May 2016 to obtain adipose and liver tissues. Brains were also collected for a separate experiment that is not part of the analyses presented here. Snakes collected from mating balls at the den were in reproductive condition, and we confirmed that females were unmated by the absence of a mating plug in the cloaca. As in Dayger and Lutterschmidt (2017) and Cease et al. (2007), we also collected migrating snakes from a road located along the migratory route approximately 1 km from the den. These snakes were migrating to summer feeding grounds and transitioning from reproductive to foraging behavior. Total sample sizes were 27 females (den: n = 16; road: n = 11) and 32 males (den: n = 16; road: n = 16).

Tissue collection

Snakes were euthanized with 250 µL if a 1% solution of sodium Brevital administrated via injection near the heart. Mass and snout-vent length (SVL) were measured for each snake and adipose and liver tissues excised. Approximately 100 mg of adipose tissue was collected from the anterior portion

of the abdominal cavity near the liver and gallbladder. Adipose tissue was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) overnight at 4°C. Tissues were then stored in 0.1 M phosphate buffer at 4°C until processed for paraffin embedding at Portland State University. Approximately 20 mm of the most posterior portion of the liver was excised and flash frozen on dry ice. Frozen tissues were kept on dry ice until arrival at the field station, where they were stored in liquid nitrogen. They were transferred to Portland State University on dry ice and then stored at -80°C until analyzed.

Adipose tissue histology

Paraffin embedding of fixed adipose tissues was conducted following the methods of Berry et al. (2014). Briefly, tissues were dehydrated in 75% ethanol for 30 min, 95% ethanol for 75 min for two incubations, and 100% ethanol for 60 min for three incubations. Tissue was then cleared in Citrisolv (product 22-143-975, Thermo Scientific) for 60 min for two incubations. Fresh solutions were used for all incubations. Tissues were impregnated with paraffin overnight at 58°C, during which paraffin wax was changed twice, approximately every seven hours. Specimens were sliced at 5 μm using a microtome and collected onto gelatin-coated microscope slides. Because of a high density of parasites within the fat bodies of some individuals, 19% of samples were re-sectioned at 15 μm. Using a thicker section allowed us to increase the proportion of adipocytes visible within each tissue section, but it did not affect the measurement of follicle area ($t = 0.86$, $df = 4$; $p = 0.440$, from a paired t-test on the mean average follicle area of 5

snakes measured at both 5 and 15 μm). Uhrig (2015) proposed that this encysted larval (metacercarial stage) diplostomid trematode likely belongs to the genus *Fibricola* based on morphology. Although investigations into possible sex-differences in parasite load are beyond the scope of this study, it is unlikely that *Fibricola* load differs between males and females because it does not significantly relate to SVL in garter snakes (Uhrig 2015). Further, pit vipers (*Bothrops jararac*) did not display any sex differences in several parasite loads among various tissues [*Caryspora* and *Sarcocystis* spp. in the gut*, Porocephalus* sp. in the lung, and *Ochetosoma heterocoelium* in the oral cavity (Grego et al. 2004)].

For each individual, we collected five sequential sections on a single slide across four series of slides (e.g., series A, B, C, D). We repeated this process 4 times, such that each tissue series comprised 4 slides; therefore within each series, a minimum of 75 μm separated the tissues collected on each slide. Using one tissue series, we measured adipocyte follicle area in one of the five tissue sections collected on each of the 4 slides within the series. Because the mean diameter of adipocytes across all red-sided garter snakes in this study was 20.81 ± 0.68 μm, it is not possible that we measured an adipocyte twice.

After collection, sections were deparaffinized for 10 min in Citrisolv for two incubations. Tissues where then rehydrated in a series of ethanol incubations followed by nanopure water and counterstained with hematoxylin and eosin (Figure 2.1). Adipocyte follicle area was measured using the Image J software Adiposoft (National Institutes of Health, Bethesda, MD, USA). Each adipocyte

follicle the software recognized was assigned a unique number. We then examined each sequential adipocyte in a section to ensure that the software had accurately measured follicles. We excluded any partial adipocytes from analysis. Where possible, we measured the follicle area of 25 cells in one tissue section per slide; we repeated this process for each slide within the series and then calculated the average adipocyte follicle area of the resulting 100 cells per snake. In the case that 100 adipocytes were not present within a tissue series, we measured as many adipocytes as possible; 11 of 59 samples had less than 100 visible adipocytes within the tissue series. The mean number of follicles measured across 9 of these individuals was 64.5 ± 9.78 s.e.m; the minimum number of follicles measured in any snake included in analysis was 32. Two males collected from the road had very little adipose tissue to initially collect. Because we could not obtain enough measurements (i.e., fewer than 20 visible follicles) even after reprocessing and slicing of adipose tissue to accurately estimate average follicle area, we excluded these males from adipocyte analyses.

Liver glycogen assay

For each individual, we homogenized 100-120 mg of liver tissue with a Bio-Gen series Pro200 (Oxford, CT, USA) in 0.5 mL of 1% HALT protease inhibitor solution (product 1862209, Thermo Scientific, Waltham, MA, USA) in assay buffer (0.936% Na_2PO_4, 0.4656% KPO_4, 1% NaCl) for 15 seconds. Liver homogenates were then centrifuged at 800 g for 10 minutes at 4 °C. We

transferred the supernatant to a fresh tube. To convert glycogen to glucose, we incubated 35 µL of supernatant with 175 µL of diluted glycogen hydrolysis enzyme (Item no. 700483, Cayman Chemical, Ann Arbor, Michigan, USA; reconstituted in 3 mL of 50 mM sodium acetate) for 30 minutes at 37 °C. We ran a glycogen standard in each digestion assay as a positive control. We then ran these digested samples in an enzymatic colorimetric assay (Item no.: 10009582, Cayman Chemical, Ann Arbor, MI, USA) following manufacturer instructions to measure glucose content. The intra-assay and inter-assay coefficients of variation were 3.08 ± 1.29 and 5.43, respectively. To validate this assay for snake plasma, we sequentially diluted both female and male supernatant and then subjected them first to the digestion assay and then to the colorimetric assay to verify that these samples displayed parallelism with the standard curve of the glucose colorimetric assay. Both diluted supernatants from female and male liver homogenates displayed parallelism with the standard curve of the glucose assay (data not shown), indicating that constituents of snake plasma do not interfere with the assay.

Statistical analyses

We utilized t-tests to determine if body condition index differed with migration status within each sex; we calculated the body condition index for female and male snakes separately because red-sided garter snakes exhibit a sexual size dimorphism, with females being larger than males. Body condition index was determined by calculating the residual from a regression of log-

transformed body mass on log-transformed SVL. To determine if adipocyte follicle area varied with sex or migration status, we utilized two-way analysis of variance (ANOVA). We controlled for potential differences related to body size by dividing each snake's mean adipocyte follicle area by its SVL (cm); we then determined if size-corrected adipocyte follicle area varied with either sex or migration status using a two-way ANOVA.

We accounted for the amount of liver homogenized by dividing glycogen content by liver mass homogenized, and then ran a two-way ANOVA. Significant main effects from these ANOVAs were further examined using Holm-Sidak multiple comparisons tests. Lastly, we used linear regressions to determine if adipocyte follicle area significantly related to liver glycogen, and if body condition index significantly related to adipocyte follicle area or liver glycogen.

Where necessary, data were square root or log transformed to meet the assumptions of parametric analysis. If data could not be transformed to meet these assumptions, non-parametric Mann-Whitney U tests were utilized. All statistics were run using Sigma Stat 12.0 (Systat Software Inc.). We set an alpha of 0.05 to determine significance; results were considered significant when $p \leq 0.05$ after rounding to two significant digits.

Results

Female snakes collected from the den had a significantly higher body condition index than migrating females collected from the road (Figure 2.2A; t =

3.72, p = 0.001). Body condition index of male snakes did not vary with collection site (Figure 2.2A; U = 97.00, p = 0.250).

During the spring, adipocyte follicle area was significantly larger in females compared to males (Figure 2.2B; $F_{1,53}$ = 46.27, p < 0.001), and this relationship persisted even after correcting for sex differences in SVL ($F_{1,53}$ = 15.44, p < 0.001; data not shown). There were no significant differences in adipocyte follicle area between den- and road-collected individuals (Figure 2.2B; $F_{1,53}$ = 0.70, p = 0.407) and the interaction between sex and collection site was statistically non-significant ($F_{1,53}$ = 0.03, p = 0.874).

Females in the spring had significantly more glycogen per mg of liver homogenized than male garter snakes (Figure 2.2C; $F_{1,55}$ = 3.89, p = 0.054). Neither migratory status (Figure 2.2C; $F_{1,55}$ = 0.24, p = 0.629) nor the interaction between migratory status and sex significantly affected liver glycogen ($F_{1,55}$ = 2.03, p = 0.160).

Adipocyte follicle area did not significantly relate to liver glycogen in females (Figure 2.3; R^2 = 0.00, p = 0.717) or males (Figure 2.3; R^2 = 0.04, p = 0.139). Neither adipocyte follicle area (Figure 2.4A; R^2 = 0.092, p = 0.124) nor liver glycogen (Figure 2.4B; R^2 = 0.00, p = 0.737) in female snakes significantly related to body condition index during the spring. Likewise, neither male adipocyte follicle area (Figure 2.4A; R^2 = 0.02, p = 0.464) nor liver glycogen (Figure 2.4B; R^2 = 0.00, p = 0.394) significantly related to body condition index. All results presented in Figures 3 and 4 are from simple linear regressions performed separately for each sex.

Discussion

This is the first investigation reporting liver glycogen in red-sided garter snakes. Our findings of no relationship between liver glycogen and migratory status are similar to investigations in migrating fish (Tudorache et al. 2007; but see Chang and Idler 1960.) and birds (McWilliams et al. 2004). In contrast to the established literature suggesting the utilization of fatty acids in many migrating animals (e.g., birds, mammals, fish, and invertebrates; Dingle 2014; Weber 2009; McWilliams et al. 2004; and Blem 1980), we do not provide evidence to suggest that adipose stores influence the decision to migrate in red-sided garter snakes. However, it is possible that measuring adipocyte follicle size is not sensitive enough to assess lipid metabolism. Our results are in line with Crews et al. (1987), who found that plasma lipid content did not change over the course of 37 days after spring emergence from simulated hibernation, the time period during which individuals migrate to summer feeding grounds. Though these two lines of evidence suggest that lipid metabolism may not influence the decision to migrate in red-sided garter snakes, measuring plasma fatty acid concentration or enzymes related to lipid metabolism in den- and road-collected snakes would provide a clearer picture of whether fatty acid metabolism is involved in deciding when to initiate migration in garter snakes. A study in tree lizards (*Urosaurus ornatus*) found that one enzyme involved in fatty acid anabolism, diacyglycerol acyltransferase, differed with reproductive condition in females but not males (Lacy et al. 2002). Because snakes located at the den were primarily performing reproductive behavior, measuring these and/or other enzymes associated with

lipid metabolism could provide further evidence as to whether adipose tissue plays a role in initiating migration in red-sided garter snakes.

Other energy-related signals such as glucose, amino acids, and other byproducts of catabolism may influence the initiation of migration. In red-sided garter snakes, total plasma protein concentrations change over 24 days post-spring emergence in field-caught males, though no clear pattern of change was evident (Crews et al. 1987). Measuring total protein likely may not provide a clear picture of amino acid metabolism because of the inclusion of other plasma proteins such as binding proteins and clotting factors. In that same study, plasma glucose levels in males did not significantly fluctuate (Crews et al. 1987); a finding similar to preliminary work in our lab that showed no significant differences in plasma glucose levels between den- and road- collected males (Maine et al. 2014). However, road-collected females displayed higher glucose concentrations than den-collected females (Maine et al. 2014), a finding that is reflected in our data because migrating females tended to have higher liver glycogen compared to den-collected females. This suggests that the factors regulating differences in liver glycogen between individuals primarily engaging in mating activities and those migrating to summer feeding grounds may be sexually dimorphic.

Sex differences in energy stores

We found significant sex differences in adipocyte follicle size and liver glycogen stores during the spring in red-sided garter snakes, with females having

significantly larger adipocyte follicles and higher glycogen per mg of liver homogenized than males. After controlling for SVL, females still had significantly larger adipocyte follicles compared to males. These data suggest that female red-sided garter snakes store energy substrates to a greater extent than males. A number of reviews have described sex-specific differences in fat distribution across body regions in mammals (Chang et al. 2018; Valencak et al. 2017; Wells 2007). In birds, female American Redstarts arrived at breeding grounds with more body fat than male conspecifics (Smith and Moore; 2003). Across taxa, no clear pattern of sexual dimorphisms emerged in liver glycogen. Storage of liver glycogen in female mammals may be more sensitive to fasting and fed cycles because fed female bats stored more liver glycogen than males, but fasted female rats had lower glycogen stores compared to males (Gustavsson et al. 2010; Freitas et al. 2009; Deuel et al. 1937). In two species of chorus frogs (*Pseudarcris crucifer* and *P. triseriata*), males had higher liver glycogen compared to females (Duffitt and Finkler 2011), as opposed to the common frog (*Rana temporaria*) that did not display any sexually dimorphic levels of liver glycogen (Smith 1950). Male Sockeye salmon (*Onocorhynchus nerka*) had higher liver glycogen compared to females (Chang and Idler 1960). It is possible that differing methodologies in the quantification of glycogen levels confounded a clear relationship in this sexual dimorphism. What is more likely is that sexually dimorphic differences in liver glycogen are dependent upon the organism's ecological niche.

Indeed, one study that investigated three snake species with differing life-history tactics found no consistent pattern in sex differences pertaining to stored forms of energy, as measured by fat and liver masses (Bonnet et al. 1998). Because the ecology of garter snakes does not perfectly align with any snake in this study but instead shares similarities with both asp vipers (*Vipera aspis*) and European whip snakes (*Coluber viridflavus*; e.g., medium vs. large bodied, viviparous vs. oviparous, fast-moving vs. slow-moving), it may be difficult to draw conclusions concerning sex-specific differences in energy stores. However, both female asp vipers and garter snakes had higher adipose and liver tissue compared to males, whereas male and female European whip snakes had equal forms of stored energy in these tissues. It's likely that certain ecological characteristics such as body size and parity contributed more to sex-specific differences of storing energy in snake species.

A possible mechanism underlying the sex-specific difference we found in energy stores of red-sided garter snakes is sex steroid hormones. Investigations into the presence of estrogen and androgen receptors in adipose tissue outside of mammals are sparse, with only one study that identified an androgen receptor in female orange-spotted groupers (Shi et al. 2012). In mammals, several studies have identified estrogen and androgen receptors in white adipose tissue (Mizutani et al. 1994; Dieudonné et al. 1998; O'Brien et al. 1998). Estrogen treatment decreased overall white adipose tissue mass, adipocyte size, and white adipose lipoprotein lipase, an enzyme that cleaves triglycerides into fatty acids for storage; ovariectomy increased these variables (Mead et al. 2002;

Mayes and Watson 2004; Jeong et al. 2007). However, in reptiles, estradiol significantly correlated with increased fat storage (Lacy et al. 2002). This discrepancy may be due to differences in estrogen receptor subtypes expressed in adipose tissue or differential effects of estrogens on subcutaneous versus visceral adipose tissue (Mayes and Watson 2004). Because the one study in reptiles that addressed the effects of estrogens on adipose tissue conflicts with mammalian studies, it is difficult to conclude if estrogens are responsible for the sexual dimorphism we observed in adipose tissue of red-sided garter snakes. Further complicating the potential effects of sex steroid hormones on adipose tissue in red-sided garter snakes is the fact that this population exhibits dissociated reproduction, in which snakes tend to have low levels of sex steroid hormones during the spring mating season (e.g., Lutterschmidt and Mason, 2009; Moore and Mason 2001; Moore et al., 2000; Krohmer et al. 1987; Whittier et al. 1987). As such, more research should be conducted in reptiles to help determine the basis of this sexual dimorphism. Compared to estrogens, the effects of testosterone on adipose tissue were more consistent across taxa: testosterone inhibited fat deposition in mammals, lizards, and birds (Ketterson et al. 1991; Lacy et al. 2002; Mayes and Watson 2004; Rynders et al. 2018; Yao et al. 2018). It is possible that the inverse relationship between testosterone and fat deposition was due to aromatization of testosterone to estradiol, but this relationship persisted even in the presence of an aromatase inhibitor (Rynders et al. 2018).

The effects of testosterone and estradiol on promoting adipose tissue breakdown and glycogen synthesis and storage, respectively, may be more complex in red-sided garter snakes, as these snakes are dissociated breeders in which peak sex steroid hormones did not coincide with peak mating behavior. However, male snakes did exhibit declining levels of testosterone during winter dormancy and [sometimes] over the course of the spring mating season (Krohmer et al. 1987; Moore et al. 2000; Moore and Mason 2001; Lutterschmidt and Mason 2009). It is possible that sex steroid hormones during the summer, fall, and winter act trans-seasonally to influence the sex differences we observed in energy metrics reported here. As stated previously, however, studies investigating the effects of sex steroid hormones on adipose tissue and energy-specific functions of the liver are lacking in snakes.

Body condition index

We found evidence to suggest that body condition index differs between snakes located at the den and those migrating to summer feeding grounds in females, but not males, a finding similar to Cease et al. (2007). Female snakes located at the den had a significantly higher body condition index than road-collected females. However, neither adipocyte follicle area nor liver glycogen varied with migration status in these snakes. Because females do not ovulate until 6 weeks post-emergence (Whittier et al. 1987), it is unlikely that the differences we observed in body condition index with migratory status in females were related to reproductive condition. In light of body condition index not

significantly relating to either adipocyte follicle area or liver glycogen content during the spring mating season (Figure 2.4), other energy and non-energy factors (i.e., water content) likely contributed more to body condition index than these energy metrics. For example, in male red-sided garter snakes, body condition index significantly related to lean body mass (i.e., muscle and skeleton weight) but not to fat body mass or liver mass (Shine and Mason 2005). In contrast, the body condition index of brown tree snakes (*Boiga irregularis*) related to total fat mass (Waye and Mason 2008). These findings illustrate that body condition index did not always represent the same stored energy substrates across species. The variation in which metabolic substrates contributed to body condition index may reflect differing ecological and environmental influences and/or metabolic needs. Therefore, species-specific validation is needed to determine which form of energy substrate most relates to body condition index. The hypothesis that body condition index does not accurately reflect stored energy content is not new (Green 2001; Peig and Green 2009; Labocha et al. 2013). Peig and Green (2009) argued for the necessity of including allometric scaling in body condition index calculations to appropriately approximate energy stores. However, using residuals from regressions of body mass on snout-vent-length are appropriate to estimate body condition in snakes because these residuals were not influenced by allometry but significantly related to measurements of fat in Burmese pythons (*Python bivittatus*; Falk et al., 2017).

In conclusion, we present evidence to suggest that body condition index related to the decision to migrate in female, but not male, red-sided garter

snakes. However, how body condition index pertained to energy substrates remains unclear in red-sided garter snakes. This research contributes to the large body of literature demonstrating sexually dimorphic storage of energy substrates across species. Because we did not find evidence to suggest that stored energy metrics influenced the decision to migrate, a physiological mechanism inducing migration in red-sided garter snakes remains elusive. Accordingly, more research will help clarify if the prioritization of fatty acid usage to fuel migration is conserved across taxa or whether energy substrate usage is dependent upon environmental or ecological conditions.

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Figures

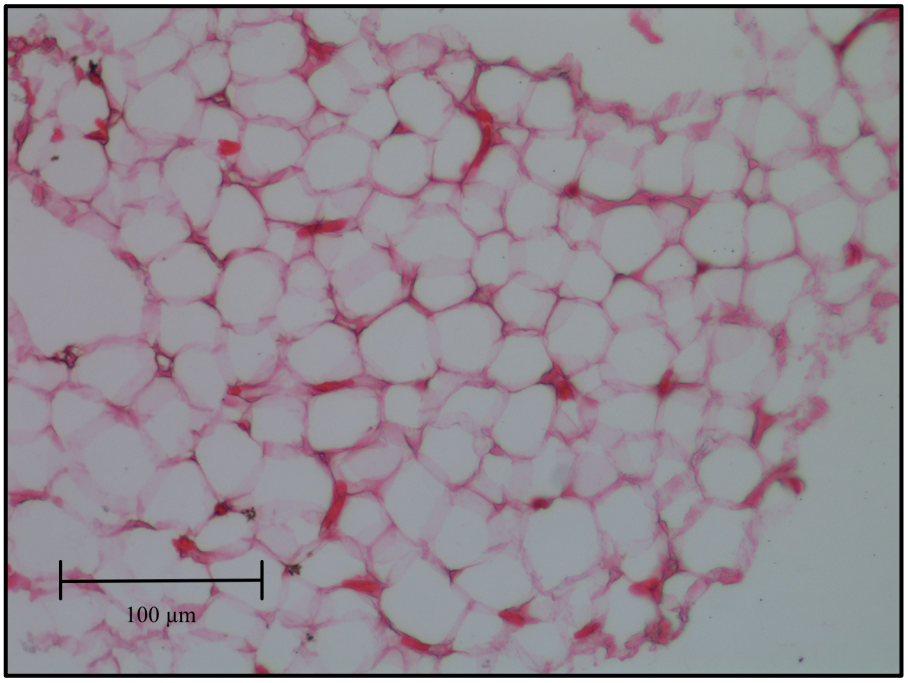

Figure 2.1. Adipose tissue section from a representative male red-sided garter snake. Tissues were hematoxylin and eosin stained and photographed at a magnification of 200x. Scale bar is shown in the lower left of the image.

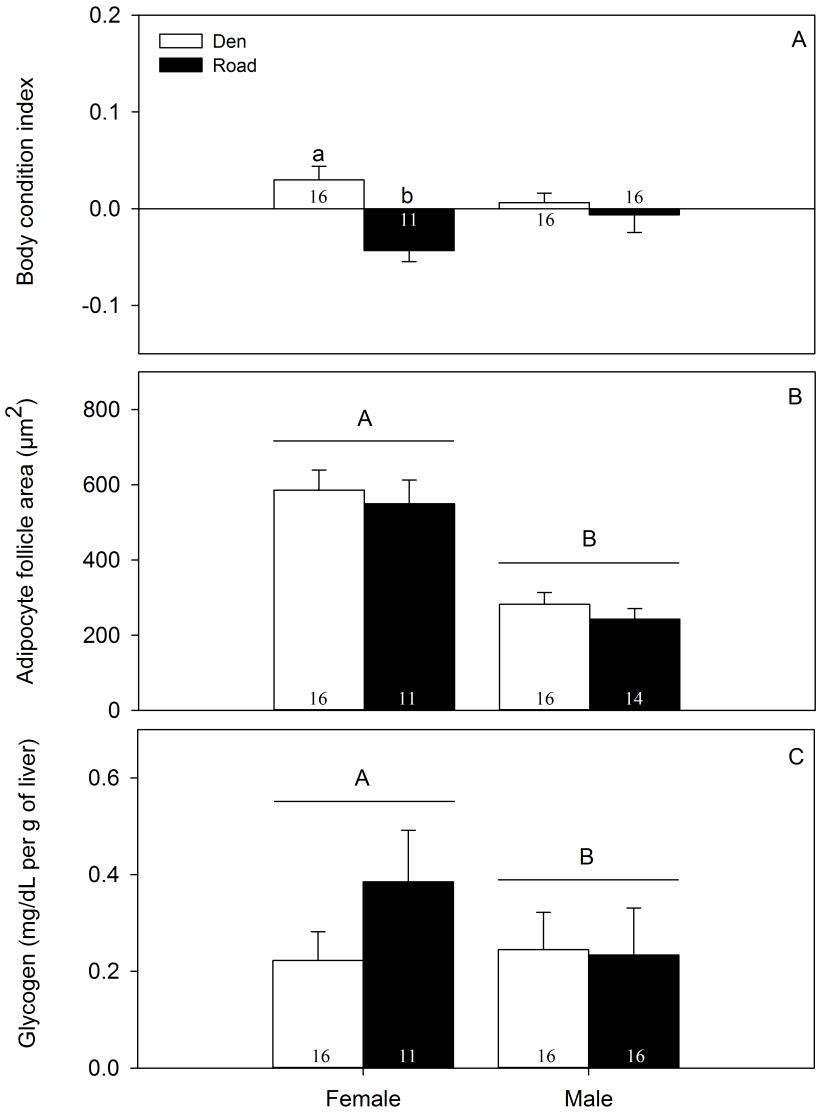

Figure 2.2. Influence of sex and migratory status on body condition index (A), adipocyte follicle area (B), and liver glycogen (C) in red-sided garter snakes (*Thamnophis sirtalis parietalis*). Non-migratory snakes were collected from the den and migrating snakes were collected from a road located ~1 km from the den along the migratory route. All data are the mean + 1 s.e.m. Letters indicate significant differences between groups; numbers along the x-axes indicate final sample sizes.

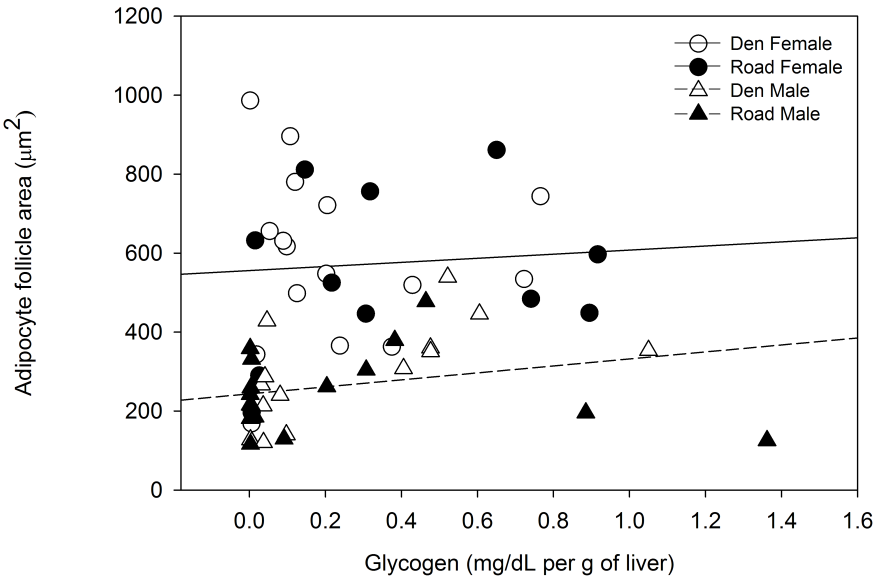

Figure 2.3. Relationship between adipocyte follicle area and liver glycogen in male and female red-sided garter snakes (*Thamnophis sirtalis parietalis*). Results are from separate linear regressions for each sex. Individuals collected from the den (open symbols) were primarily engaging in mating behavior, whereas individuals that initiated migration to summer feeding grounds were collected from a road (solid symbols) approximately 1 km from the den. Because we found no significant differences in either variable between den- and road-collected individuals, we collapsed migratory status for these analyses. Adipocyte follicle area did not significantly relate to liver glycogen content in either female or male red-sided garter snakes (see text for further statistical details).

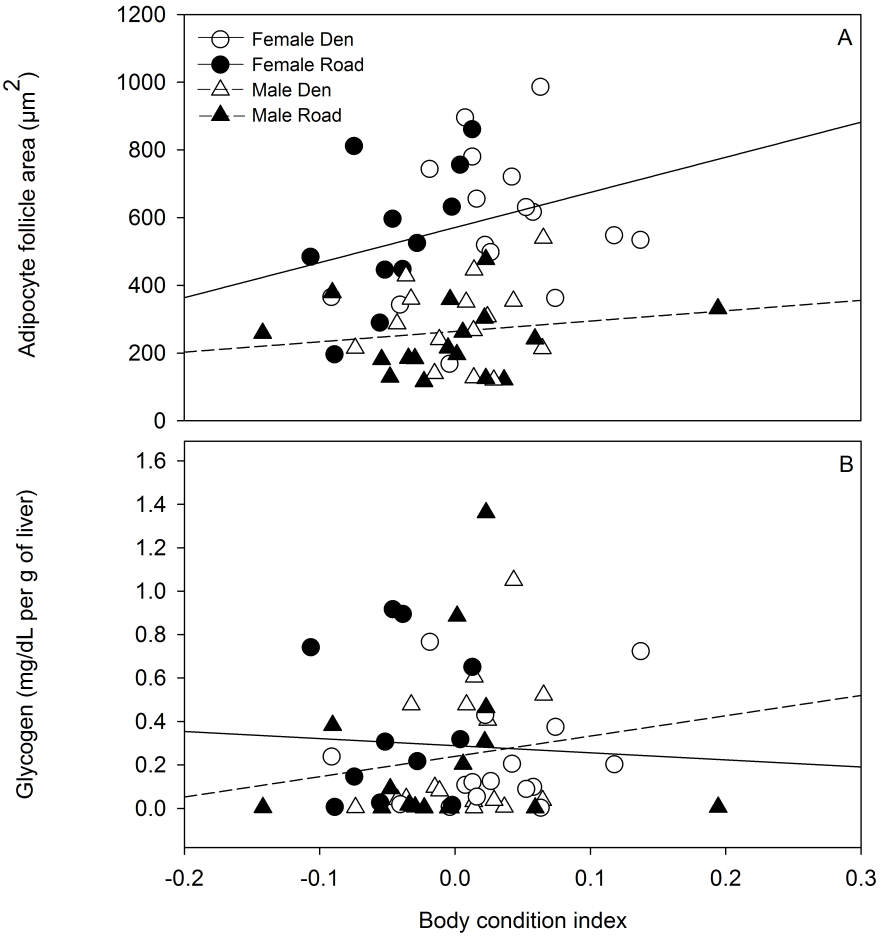

Figure 2.4. Relationship between adipocyte follicle area (A) or liver glycogen (B) and body condition index in male and female red-sided garter snakes (*Thamnophis sirtalis parietalis*). Results are from simple linear regressions run separately for each sex. Individuals collected from the den (open symbols) were primarily engaging in mating behavior, whereas individuals that initiated migration to summer feeding grounds were collected from a road (solid symbols) approximately 1 km from the den. Body condition index was determined separately for female and male snakes by calculating the residual from a regression of log-transformed body mass on log-transformed snout-vent length. Because we found no significant differences in adipocyte follicle area or liver glycogen between den- and road-collected individuals, we collapsed migratory status for these analyses. Body condition index did not significantly relate to either adipocyte follicle area or liver glycogen in female or male red-sided garter snakes (see text for further statistical details).

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Chapter 3

Exogenous leptin promotes reproductive behavior during aphagia in red-sided garter snakes (*Thamnophis sirtalis parietalis*)

Abstract

Despite the established dichotomy between investment in either reproduction or self-maintenance, a hormonal mechanism that influences an organism's decision to prioritize these behaviors remains elusive. The peptide hormone leptin is a likely candidate because in many species it is secreted from adipocytes in proportion to the amount of stored fat. Although most studies suggest that leptin stimulates reproduction, the actions of leptin can be context-dependent. Leptin increases sexual behavior in fed individuals, but inhibits sexual behavior in food-restricted individuals. We investigated if exogenous leptin influences sexual behavior in red-sided garter snakes (*Thamnophis sirtalis parietalis*) experiencing a predictable bout of aphagia during the mating season. We injected two doses of recombinant murine leptin over three days. Males were subjected to three mating trials, one on each day of injections, while females were subjected to one mating trial on the last day of injections. We found no evidence to suggest that leptin influenced latency to copulate or duration of copulation. We did find that leptin increases appetitive (i.e., courtship score) and consummatory (i.e., number of copulations) sex behavior in males, but only consummatory sex behavior in female snakes. Therefore, we present a potential

sexual dimorphism in the effects of leptin on sex behavior in red-sided garter snakes. Because leptin promotes reproductive behavior in non-feeding garter snakes, these findings do not align with research on food-restricted mammals. Further investigations into how leptin affects sexual behavior in snakes exposed to food-restriction manipulations would clarify if the role of leptin is evolutionarily divergent.

Introduction

All organisms must decide to invest in either reproductive or self-maintenance (e.g., feeding or foraging) activities, as organisms often cannot perform these activities simultaneously. Despite the thoroughly established tradeoffs associated with investment in either reproduction or self-maintenance, a physiological mechanism that influences an organism's decision to prioritize one behavior over another remains elusive. A multitude of evidence suggests that energy substrates, specifically fat stores, positively relate to reproduction (Doughty and Shine, 1998; Harlow et al., 2002; Lu et al., 2012; Smith and Moore, 2003; Wapstra and Swain, 2001). Further, insufficient energy stores can prematurely end reproductive activities (Groscolas et al., 2008). Because reproduction is highly energetically expensive, an energy-related signal likely exists to indicate when it is appropriate to prioritize reproductive over other self-maintenance activities such as foraging and feeding behaviors.

A likely candidate to signal the appropriate time to perform reproductive activities is the peptide hormone leptin, as it is secreted from adipocytes in

proportion to the amount of fat in numerous mammalian species (Delavaud et al., 2000; Frederich et al., 1995; Fuglei et al., 2004). However, during the prehibernation period in little brown bats (*Myotis lucifiugus*), leptin secretion is dissociated from body fat percentage (Kronfeld-Schor et al., 2000). Such a dissociation may be necessary to ensure the accumulation of sufficient energy stores for survival during life-history stages such as hibernation or migration. Despite this dissociation in some species, leptin decreases with fasting and increases with refeeding in a few fish species (Garcia-Suarez et al., 2018; Volkoff, 2015; Won et al., 2012) and a wide range of mammals (Chelikani et al., 2004; Daniel et al., 2002; Delavaud et al., 2000; Walker et al., 2005; Weigle et al., 1997; Widmaier et al., 1997).

While comparatively little research has focused on the function of leptin in ectotherms other than fishes, leptin has been identified in various tissues of a few reptiles. Preliminary results identified the leptin gene and its receptor in the Green anole (*Anolis carolinensis*; reviewed in Denver et al., 2011). The leptin protein was identified in several lizard tissues including plasma, whole blood, brain, and stomach (Spanovich et al., 2005; Muruzàbal et al., 2002; Paolucci et al., 2001; Niewiaroski et al., 2000). To date, only one study has examined the leptin protein in snakes, and it was found in the stomach of natricine water snakes (*Natrix maura*; Muruzàbal et al., 2002). In some non-mammalian species including fishes, leptin mRNA is expressed in tissues other than adipose (e.g., liver; Denver et al., 2011; Muruzàbal et al., 2002). Further, leptin does not accurately signal stored energy substrates in fishes, and therefore species-

specific validations are necessary to determine what energetically-relevant information leptin conveys (Londraville et al., 2014).

Regardless of the tissue that produces and secretes leptin, numerous lines of evidence from an array of taxa (e.g., mammals, reptiles, fishes) suggest that leptin promotes reproductive behavior and physiology (Finn et al., 1998; Peyon et al., 2001; Putti et al., 2009; Schneider et al., 2007; Weil et al., 2003). For example, whole blood leptin decreases after reproductive activities cease eastern fence lizards (*Sceloporus undulatus*; Spanovich et al., 2005), and plasma leptin is highest at the start of the reproductive season in female Italian wall lizards (*Podarcis sicula*; Paolucci et al., 2001). However, the role of leptin can be context-dependent, as exgenous leptin causes the initiation of a second clutch in one study on great tits (*Parus major*), but not in another using identical methods (Lõhmus and Björklund, 2009; Marvelde and Visser, 2012; but see Friedman-Einat and Seroussi, 2019, 2014; Londraville et al., 2014; and Prokop et al., 2014 for further dicussion on avian leptin). Further highlighting the context-dependent role of leptin is the finding that leptin promotes reproductive behavior in fed female Syrian hamsters (*Mesocricetus auratus*) but inhibits reproductive behavior in food-deprived females (Wade et al., 1997). For these reasons, we aimed to determine the influence of leptin on reproductive behavior in a species that is aphagic prior to and during the mating season.

Northern populations of red-sided garter snakes (*Thamnophis sirtalis parietalis*) exhibit an annual life-history cycle that allows the investigation of leptin's role in reproduction in the absence of feeding behavior. Snakes

overwinter for approximately 8 months in underground hibernacula and upon spring emergence engage in an intense and short mating period all while being aphagic (Gregory and Stewart, 1975). During the spring mating season, red-sided garter snakes exhibit robust and stereotyped mating behaviors (e.g., Crews et al., 1984). After varying amounts of time spent at the den performing mating behaviors, snakes migrate up to 17 km to summer feeding grounds to replenish energy stores (Gregory, 1977; Gregory and Stewart, 1975). Snakes leave the summer feeding grounds to migrate back to overwintering sites in the fall, and once again become aphagic. Therefore, investigating if leptin promotes or inhibits reproduction in an organism experiencing predictable bouts of aphagia will provide insight into whether the role of leptin differs with an animal's ecology.

In the present study, we aimed to determine how leptin influences both appetitive and consummatory sex behaviors in red-sided garter snakes. Appetitive sexual behavior describes an individual's motivation to gain access to sexual partners. In contrast, consummatory sexual behavior concerns behaviors associated with the act of copulation and often includes measurements such as the number of mounts, intromissions, and ejaculations (Beach, 1956). Because different regions of the brain control appetitive and consummatory sexual behaviors (Balthazart and Ball, 2007), we wanted to investigate the effect of leptin on both, as prior evidence suggests that leptin can differentially affect consummatory versus appetitive sexual behaviors in female rats (García-Juárez et al., 2011). In red-sided garter snakes, we measured appetitive sex behavior by quantifying reproductive behavior using ethograms. In male snakes, we

measured the following consummatory sexual behaviors: number of copulations, duration of copulation, and copulatory plug mass. In females, we measured the proportion of females that copulated and duration of copulation. We hypothesized that leptin treatment affects both appetitive and consummatory sexual behaviors in red-sided garter snakes, and predicted that leptin would increase sexual behaviors.

Material and methods

Experiments were conducted on field-caught red-sided garter snakes (*Thamnophis sirtalis parietalis*) from Inwood, Manitoba, Canada. Female snakes were studied over two consecutive years during the May 2016 (n = 29) and 2017 (n = 90) spring mating seasons. Male snakes (n = 72) were collected during May 2017. The Portland State University Institutional Animal Care and Use Committee (protocol numbers 40 and 54) and Manitoba Department of Sustainable Development (collecting permit WB18801) approved all experimental procedures. For all snakes, we measured mass and snout-vent length (SVL) to ensure these variables did not differ among treatment groups. After completion of the experiments, we released snakes at their initial capture site.

Leptin injections

In 2016, female snakes were assigned to one of two treatment groups: vehicle or high leptin dose. Snakes collected in 2017 were randomly assigned to one of three treatment groups: vehicle, low leptin dose, or high leptin dose. Recombinant murine leptin was purchased from the National Hormone and

Pituitary Program at Harbor-UCLA Medical Center, Torrance CA. Similar to French et al. (2011), we used doses of 0, 7, or 70 µg leptin for females; males were injected with 0, 3, or 30 µg leptin. Female doses were higher than males because of a sexual size dimorphism in red-sided garter snakes where females are larger and heavier than their male counterparts. Injection volume was 0.1 mL of solution regardless of leptin treatment. Ampoules of recombinant leptin were reconstituted with 0.1 M phosphate buffer following manufacturer instructions, which we also used as vehicle injections. Snakes were injected intraperitoneally once a day between 0900 and 1000 h for three consecutive days.

Female reproductive behavior

One hour following the third leptin injection, we subjected female snakes to a single mating trial, as females become unattractive after mating (Garstka et al., 1982; O'Donnell et al., 2004). Mating trials occurred in 1 x 1 x 1 m^3 cloth arenas where we provided females with the opportunity to mate for 60 min in the presence of 25 males in 2016 or 15 males in 2017. When a mating occurred, we recorded latency to copulate and then carefully removed the copulating pair to a separate arena to measure duration of copulation. We developed an ethogram (Table 3.1) to score the behavior of each female every 20 min during the 60-min mating trial or until copulation occurred. In some cases (n = 29), females mated before we could score their mating behavior, and these females were not included in analysis of behavior scores.

Male reproductive behavior

To determine if leptin affected male reproductive behavior, we conducted a three-hour long mating trial in 1 x 1 x 1 m^3 cloth arenas on each day of injections. Trials were initiated one hour after injections. Equal numbers of males from each treatment group (n=4 per group for a total of 12 males per arena) were introduced to a single, sexually attractive female red-sided garter snake. We deemed females attractive if they elicited courtship from at least 5 males.

During mating trials, we measured courtship scores once every 20 min using a previously described ethogram (Table 3.1; Crews, 1976; Lutterschmidt et al., 2004). When a mating occurred, we recorded latency to copulate and the copulating pair was carefully removed from the mating arena and placed in a separate arena to measure duration of copulation. At cessation of copulation, we placed the mated female in a cloth bag until we could remove and weigh copulatory plugs later that day to determine if exogenous leptin affected copulatory plug mass; the mated male was moved to his home arena. After removal of a copulating pair, we introduced a new female into the arena along with a new, non-experimental male to maintain an equal number of males in all arenas. We taped the cloaca of the non-experimental male with medical adhesive tape to prevent him from copulating. This procedure limited the number of copulations a male could perform to 1 per day. In the event a mating did not take place in an arena within 60 min, we removed the unreceptive female (e.g., Dayger et al., 2013) and introduced a new, sexually attractive female into the mating arena.

Statistical analyses

Where necessary, data were either log- or square root-transformed to meet the assumptions of parametric tests. If data could not be transformed to adhere to assumptions, we used non-parametric tests. All statistics were run in Sigma Stat 12.5 (Systat Software, Inc.).

To verify that our ethogram of female mating behavior (Table 3.1) accurately assessed a female's interest in copulation, we used a t-test to determine if the mating behavior score differed between females that mated and those that did not mate during behavior trials. We excluded any scores of 6 (i.e., female copulates) in this analysis as only females that mated could achieve this score. To ensure no differences existed in female morphometrics between years and among treatment groups, we ran a two-way analysis of variance (ANOVA) on both mass and SVL.

We used a one-way ANOVA to determine if treatment with leptin affected average mating behavior score; we did not include year in this analysis because we developed the female behavior ethogram in 2017 and therefore there were no mating behavior scores from 2016. To determine if leptin affected the proportion of females that mated, we used a 3 x 3 Chi-square test. Because we observed the same mating proportions across years (from a Chi-square test on controls between 2016 and 2017: $X^2 = 0.11$, $p = 0.746$; and a Fisher's exact test on high leptin-dosed females between 2016 and 2017: $p = 1.00$), we combined data for analysis to increase sample size. As post-hoc tests, we used 2 x 2 Chi-square tests to determine which treatment groups significantly differed. In those females that mated (i.e., were receptive), we determined if exogenous leptin affected

latency to copulate (i.e., proceptivity; Dayger et al., 2018) and the duration of copulation using a two-way ANOVA with treatment and year as between-subjects factors. An unbalanced experimental design, with 2 leptin treatments in 2016 and 3 leptin treatments in 2017, precluded us from running an interaction between year and treatment group for these analyses.

As in females, we ensured that no significant differences existed in male morphometrics among groups using separate one-way ANOVAs. We utilized a two-way repeated measures ANOVA with treatment as the between-subjects factor and day as the within-subjects factor to determine if leptin affected average courtship score. To determine if exogenous leptin affected the number of matings a male achieved, we utilized a one-way ANOVA. Within males that mated, we also utilized one-way ANOVAs to determine if leptin affected latency to copulate, duration of copulation, and copulatory plug mass. We analyzed these response variables only for the first mating a male performed regardless of the day that the mating occurred because the low sample sizes of males that performed multiple matings (second matings: n = 4, 5, 9; third matings: n = 0, 1, 2 for control, low leptin, and high leptin individuals, respectively) may not provide statistically meaningful results.

Results

Female reproductive behavior

In females, neither mass nor SVL differed significantly by year (Mass: $F_{1,115} = 2.54$, $p = 0.114$; SVL: $F_{1,115} = 0.26$, $p = 0.615$) or treatment group (Mass:

$F_{2,115}$ = 0.56, p = 0.570; SVL: $F_{2,115}$ = 0.14, p = 0.873). Females that went on to mate had a significantly higher average mating behavior score prior to mating (i.e., prior to achieving a score of 6) than females that did not mate (Figure 3.1, U = 222.5, p = 0.001), verifying that our female ethogram (Table 3.1) accurately assessed a female's interest in mating.

Average mating behavior score was not influenced by leptin treatment (from a nonparametric one-way ANOVA on ranks; Table 3.2). However, exogenous leptin significantly increased the proportion of female snakes that mated (Table 3.2, Figure 3.2A). Two-way Chi-squared tests for pairwise comparisons between treatments revealed that only the control and high leptin treatment groups differed significantly in the proportion of females mating (Figure 3.2A; X^2 = 7.17, p = 0.007). Of those females that mated, neither treatment nor year influenced latency to copulate (Table 3.2, Figure 3.2B). There was no effect of leptin treatment on duration of copulation (Table 3.2, Figure 3.2C), but females tested in 2017 had a longer duration of copulation compared to females in 2016 (Table 3.2).

Because the differences in copulation duration observed between years may reflect variation in environmental conditions and/or resource availability, we wanted to examine potential factors that could explain the difference in copulation duration between years. Body condition index can be an integrative proxy for environmental conditions and resource availability (McKinnon et al., 2015). As such, we wanted to determine if female body condition index differed between years and/or influenced responses to exogenous leptin. We calculated

body condition index as the residual from a regression of log-transformed body mass on log-transformed SVL in females collected in each of 2016 and 2017. We converted each numerical body condition index into categorical data by assigning females with a residual below 0 as negative and above 0 as positive body condition (Dayger et al., 2013). After running a two-way ANOVA, we found that the mean body condition index of females in 2017 (mean 0.005 ± 0.005 SE) was significantly higher than that in 2016 (-0.019 ± 0.010; Table 3.2). Female body condition index did not differ significantly among treatment groups (Table 3.2). We then re-analyzed the duration of copulation data with body condition index instead of year. Leptin treatment, body condition index, and the interaction between treatment and body condition index did not significantly affect duration of copulation (Table 3.2).

Male reproductive behavior

Differences in male morphometrics were non-significant among treatment groups (Mass: $F = 0.24$, $p = 0.786$; SVL: $H = 2.09$, $p = 0.352$). Leptin treatment and day significantly affected the average courtship score of males (from a two-way repeated measures ANOVA; Table 3.3, Figure 3.3). The interaction between treatment and day was non-significant (Table 3.3). Pairwise comparisons from a Student-Newman-Keuls test revealed males treated with the low and high leptin dose had significantly higher average courtship scores compared to controls (Figure 3.3). Average courtship scores significantly decreased after day 1 for males dosed with vehicle and high leptin, but not in low leptin-dosed individuals

(Figure 3.3). The mean number of copulations achieved by male snakes significantly differed among treatment groups, with males receiving the high leptin dose performing significantly more copulations compared to controls (from a one-way ANOVA; Table 3.3, Figure 3.4). Of the males that mated, the only snakes that performed 3 matings received injections with exogenous leptin (Low leptin: n = 1; high leptin: n = 2). There was no significant effect of leptin treatment on latency to copulate, duration of copulation, or copulatory plug mass (Table 3.3; Figure 3.5A-C).

Discussion

We present evidence to suggest that leptin promotes reproductive behavior in both male and female red-sided garter snakes. As such, this is the first direct examination of whether leptin differentially affects appetitive and consummatory sex behaviors in males and females of any vertebrate species. Our results do not indicate that leptin universally increases reproductive behavior, as we found no evidence to suggest that leptin influenced latency to copulate or duration of copulation. Our results are consistent with research in fed mammals (García-Juárez et al., 2012, 2011; Schneider et al., 2007; Wade et al., 1997). Because these snakes are aphagic during the spring mating season (Crews et al., 1987; Gregory and Stewart, 1975; O'Donnell et al., 2004), our results do not align with research suggesting that leptin inhibits reproduction in organisms that are not feeding (Wade et al., 1997). For example, treatment with leptin in food-deprived female Syrian hamsters significantly decreased lordosis

duration, a measure of sexual receptivity, compared to vehicle-treated and food-deprived female Syrian hamsters. This suggests that multiple signals convey an organism's energy status, and a mismatch in signals may inhibit reproduction. Further research examining the effects of leptin on fed and food-deprived reptiles, particularly in other species of garter snakes, would help clarify the relationship between leptin, sexual behavior, and feeding status.

We also present possible sex differences in the effects of leptin on reproductive behavior in red-sided garter snakes. While leptin increases both appetitive (e.g., courtship score) and consummatory (e.g. number of copulations) sex behavior in male snakes, we only find evidence to suggest that leptin increased consummatory sex behavior in female snakes. For appetitive sex behavior in males, a low dose of leptin prevented the decrease in courtship score on days 2 and 3 that we observed in vehicle- and high leptin-treated males. Because leptin evokes different behavioral responses at low and high doses, it is likely that leptin does not elicit a linear dose-response curve for courtship score in these snakes as is the case for some mating behaviors in female rats (Fox et al., 2000; García-Juárez et al., 2011). In contrast to our findings in males, leptin did not affect mating behavior score in females, a measure of appetitive sex behavior. It is possible that the average mating behavior score was under-estimated in this experiment, because some females mated very quickly, and therefore prior to recording a mating behavior score. This may have prevented us from observing a statistically significant effect of leptin on female appetitive sex behavior. However, it is also possible that leptin did not affect appetitive sex

behavior (e.g., mating behavior score, latency to copulate, etc.) in female snakes, as is the case in female rats (Fox et al., 2000; García-Juárez et al., 2011). We did find that leptin increased consummatory sex behavior in females, as leptin increased the proportion of individuals that mated. How leptin promotes sexual behavior in red-sided garter snakes is unclear.

Unfortunately, only one published paper reports the preliminary identification of the leptin gene in any reptile (i.e., the Green anole; reviewed by Denver et al., 2011), despite numerous predicted mRNA sequences available in the NCBI's GenBank database. As discussed earlier, identification of the leptin protein has occurred in several reptilian tissues: plasma, whole blood, brain, and stomach (Spanovich et al., 2005; Muruzàbal et al., 2002; Paolucci et al., 2001; Niewiaroski et al., 2000). Although the mechanism by which leptin exerts its effects to induce the behavioral changes we observed are not clear, leptin must bind to a receptor to evoke physiological changes (Myers et al., 2008). The mammalian leptin receptor is present in an array of tissues including the hypothalamus, pituitary, and gonads (Margetic et al., 2002). Investigations into the reptilian leptin receptor are sparse: one published study identified the leptin receptor protein in thyroid tissue of Italian wall lizards (Sciarrillo et al., 2005), and preliminary results suggest leptin receptor mRNA is present in brain tissue of Green anoles (Denver et al., 2011). Because the few ectotherms examined to date express the long form of a hypothalamic leptin receptor (Denver et al., 2011), the serpentine hypothalamus likely also contains the leptin receptor.

The long form of the leptin receptor is the most commonly expressed isoform in the mammalian hypothalamus and is the only isoform that activates cell signaling pathways (e.g., Denver et al., 2011; Landry et al., 2013; Margetic et al., 2002; Zabeau et al., 2003, Villanueva ad Myers, 2008). Within the hypothalamic preoptic area of mice, the expression of the long-form leptin receptor gene is highest in the medial preoptic area relative to other preoptic regions (e.g., lateral preoptic area, paraventricular nucleus; Scott et al., 2009). This, in conjunction with the idea proposed by Balhazart and Ball (2007) that different preoptic regions influence appetitive and consummatory sex behaviors, may provide a physiological mechanism (i.e., through differences in leptin receptor density) that explains how appetitive and consummatory sex behaviors are differentially modulated by leptin.

Numerous reviews describe the multitude of pathways that are activated when leptin binds to its receptor [e.g., Janus kinase /signal tranducers and activators of transcription (JAK/STAT), mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR1), and phosphoinositide-3 kinase (PIK3)] (e.g., Denver et al., 2011, Margetic et al., 2002; Zabeau et al., 2003, Villanueva ad Myers, 2008). However, the majority of these reviews focus heavily on signaling cascades pertaining to energy balance rather than reproduction, and not all pathways are implicated in leptin promoting reproduction. For example, leptin may influence reproduction in the hypothalamus by suppressing agouti-related peptide/neuropeptide Y (AgRP/NPY) neurons, thereby indirectly promoting the release of gonadotropin-releasing hormone (GnRH) by removing

the inhibitory influence of NPY on GnRH (Egan et al., 2017; Landry et al., 2013). This mechanism may explain our findings of leptin increasing reproductive behavior, because exogenous NPY inhibits sexual behavior in male red-sided garter snakes (Morris and Crews, 1990).

Another mechanism by which leptin could exert its effects on reproductive behavior is through the nitric oxide pathway. Leptin increases the number of phosphorylated (i.e., activated) nitric oxide synthase-immunoreactive neurons in the hypothalamus, thereby increasing the production of nitric oxide (Bellefontaine et al., 2014). Further, inhibitors of the nitric oxide pathway prevent the stimulatory effects of leptin on receptivity in female rats (García-Juárez et al., 2012). In red-sided garter snakes, initial studies indicate that nitric oxide synthase-immunoreactive neurons are present in areas of the brain associated with regulation of reproductive behavior (Krohmer and Lutterschmidt, 2011). Therefore, the positive effects of leptin on reproductive behavior we present here could be a product of leptin activating nitric oxide signaling pathways. Although it is likely that leptin exerts its effects through binding to the leptin receptor, we cannot rule out the possibility the effects of leptin in this study resulted from an alternative and non-receptor-mediated pathway.

An unexpected, interesting finding was the significantly shorter duration of copulation of females in 2016 compared to 2017. We did not find evidence to suggest that body condition index explained this difference, even though females in 2017 had a significantly higher body condition index than females in 2016. Other potential factors that could influence copulation duration include male

physiology regarding copulatory plug deposition, environmental conditions (e.g., ambient temperature), or population density. The large basal spines on the hemipenes of male red-sided garter snakes may preclude females from detaching from intromission prematurely. Indeed, ablating the basal spines significantly decreases the duration of copulation in red-sided garter snakes (Friesen et al., 2014). The finding that females under anesthesia mated longer than females not subjected to anesthesia further suggests that, given the opportunity, females will end intromission early (Friesen et al., 2014). Regardless of which sex is the primary driver of copulation duration in red-sided garter snakes, the question of why we observed this difference in copulation duration between years still persists.

Research into the effects of ambient temperature and density on copulation in reptiles is inconclusive (Olsson et al., 2011; Shetty and Shine, 2002; Shine et al., 2000) and lacking, respectively. One study that investigated the influence of male density on copulation duration may provide insight into our findings of a shorter copulation duration in 2016 when mating trials consisted of a higher density of males. This study found that male Australian Polydesmidan millipedes (*Gigantowales chisholmi*) exposed to a greater number of males prior to one-on-one mating trials copulated for a shorter duration compared to males kept in isolation (Holwell et al., 2016). Because the Australian Polydesmidan millipede and red-sided garter snake mating systems are both characterized by scramble competition (e.g., Duvall et al., 1993; Friesen et al., 2013; Thornhill and Alcock, 1983), the findings of Holwell et al. (2016) are likely relevant to the red-

sided garter snake system. Further investigations into how traits such as latency to copulate and duration of copulation are affected by density-dependence would provide a more comprehensive view of potentially more nuanced factors contributing to sexual conflict. As in most facets of biology, there is likely no one primary determinant influencing duration of copulation. More likely, there are culminating and interactive effects of multiple factors that influence copulation duration and other sexual processes.

The major findings of this study demonstrate that exogenous leptin increases reproductive behavior in red-sided garter snakes during a long-term bout of aphagia. Investigating the role of leptin on reproductive behavior in a species of garter snake that is phagic during the mating season, which would allow food-restriction manipulations, would provide further insight into how the role of leptin in reptiles compares to that in fed and fasted mammals. Exogenous leptin does not universally promote reproduction in red-sided garter snakes, as we did not observe differences in all measured variables (i.e., female mating behavior score, latency to copulate, and duration of copulation). It is possible that leptin induces these behavioral changes through a variety of mechanisms after leptin binds to its receptor. Because neither leptin nor its receptor have been identified in snakes, identifying leptin and leptin receptor genes, along with characterizing the role of leptin in other reptilian species, would further clarify to what extent the role of leptin varies across species.

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Tables and Figures

Table 3.1. Ethograms of sex behavior of female and male red-sided garter snakes (*Thamnophis sirtalis parietalis*)

Score	Female behavior description	Male behavior description
0	Female is moving rapidly away from courting males	No reproductive behavior
1	Female is moving slowly	Male investigates female, increased tongue-flick rate
2	Female's body, including head and tail, is flattened to ground	Male chin rubs female with rapid tongue flicks
3	Stationary female allows males to manipulate her limp tail	Male aligns body with female
4	Stationary female actively elevates tail, responds to male manipulation of tail	Male actively tail searches and attempts cloacal apposition and copulation with female; possible caudocephalic waves
5	Female rolls her body slightly to the side and gapes her cloaca	Male copulates with female
6	Female copulates	-

The female ethogram was developed for this experiement, while male behavior descriptions are as in Lutterschmidt et al., 2004 (modified from Crews et al., 1984 and Moore et al., 2000)

Table 3.2. Statistical results from analyses of the effects of exogenous leptin on female reproductive behavior in red-sided garter snakes (*Thamnophis sirtalis parietalis*).

Response variable	Factors	Test statistic	p-value
Mating behavior score	Leptin treatment	$H = 4.08$	0.130
Proportion of mated females	Leptin treatment	$X^2 = 8.51$	**0.014**
Latency to copulate	Leptin treatment	$F_{2,72} = 2.26$	0.112
	Year	$F_{1,72} = 0.14$	0.711
Duration of copulation	Leptin treatment	$F_{2,72} = 1.20$	0.307
	Year	$F_{1,72} = 7.06$	**0.010**
Body condition index	Leptin treatment	$F_{2,115} = 0.72$	0.491
	Year	$F_{1,115} = 4.98$	**0.028**
Duration of copulation	Leptin treatment	$H_{2,70} = 1.41$	0.486
	Body condition	$H_{1,70} = 0.27$	0.606
	Leptin*Body condition	$H_{2,70} = 1.54$	0.462

Because of the significant difference in duration of copulation between years, we used body condition index as a proxy for environmental conditions and re-analyzed the data (indented Response variables). Bold font indicates $p < 0.05$.

Table 3.3. Statistical results from analyses of the effects of exogenous leptin on male reproduction in red-sided garter snakes (*Thamnophis sirtalis parietalis*).

Response variable	Factors	Test statistic	p-value
Courtship score	Leptin treatment	$F_{2,138} = 3.64$	**0.031**
	Day	$F_{2,138} = 14.41$	**< 0.001**
	Leptin*Day	$F_{4,138} = 2.05$	0.090
Mean no. of copulations	Leptin treatment	$F = 4.09$	**0.022**
Latency to copulate	Leptin treatment	$F = 0.77$	0.469
Duration of copulation	Leptin treatment	$F = 0.06$	0.941
Copulatory plug mass	Leptin treatment	$H = 0.09$	0.958

Bold font indicates $p < 0.05$. One male achieved intromission and immediately detached from the female; we therefore included him in the latency to copulate analysis but excluded him from duration of copulation and copulatory plug mass. Analyses for latency to copulate, duration of copulation, and copulatory plug mass are of first matings only.

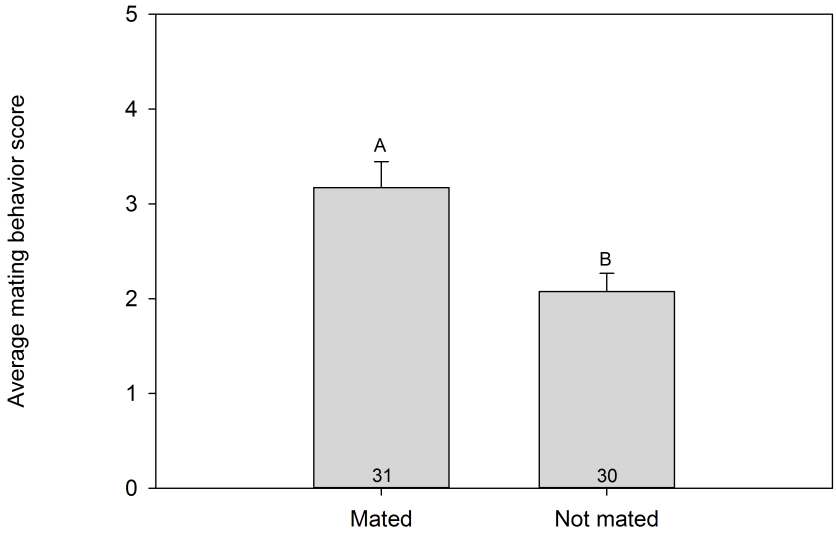

Figure 3.1. Verification that the mating behavior ethogram for female red-sided garter snakes accurately assesses a female's interest in copulation. Females that went on to mate displayed significantly higher average mating scores prior to mating than females that did not go on to mate during a 60 min mating trial. Scores were compared prior to mating, and therefore these data did not include any scores of 6 (i.e., mating). Data are mean + 1 s.e.m. Letters indicate significant differences between treatment groups.

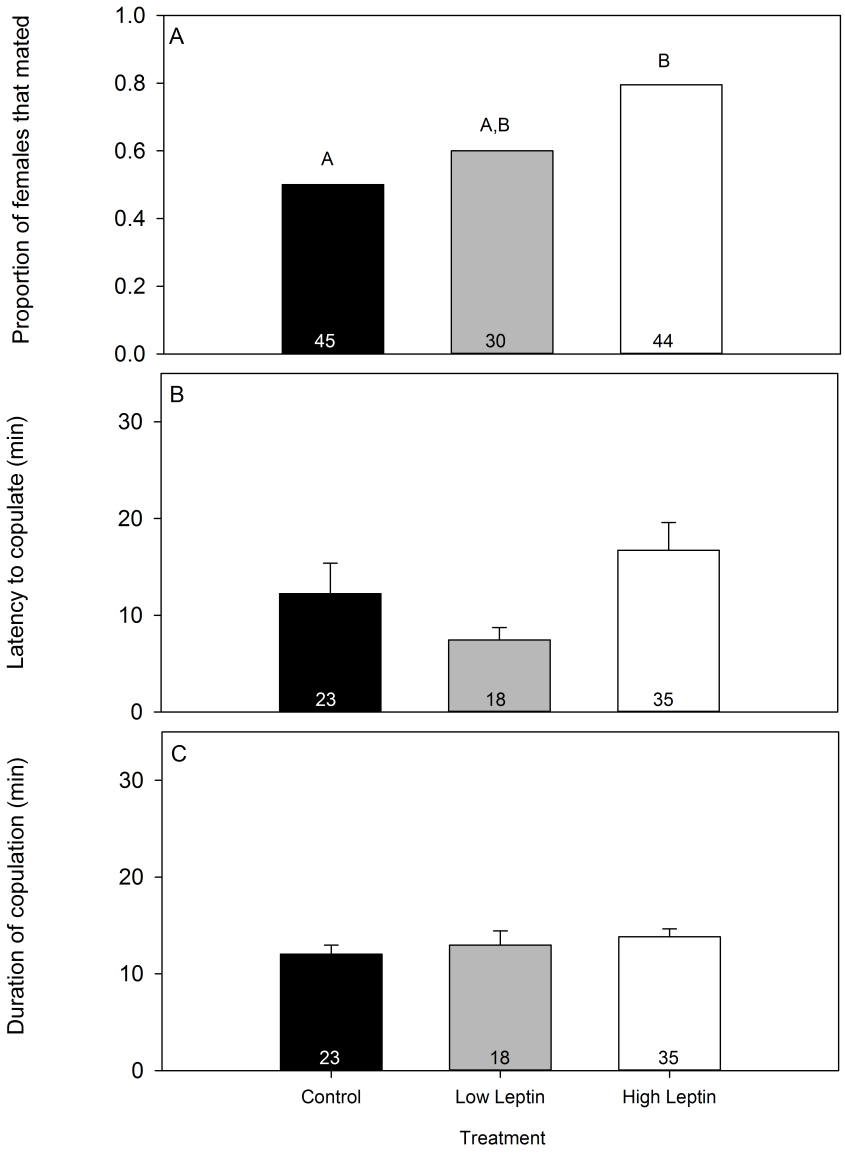

Figure 3.2. The effect of exogenous leptin on (A) the proportion of females that mated, (B) latency to copulate, and (C) duration of copulation in female red-sided garter snakes. Snakes were injected daily with 0, 7, or 70 µg of mouse recombinant leptin (Control, Low leptin, or High leptin, respectively) for 3 days prior to mating trials. Only females that copulated were included in panels B and C. Numbers along the x-axes represent sample sizes in each group. Letters indicate statistically significant differences among treatment groups.

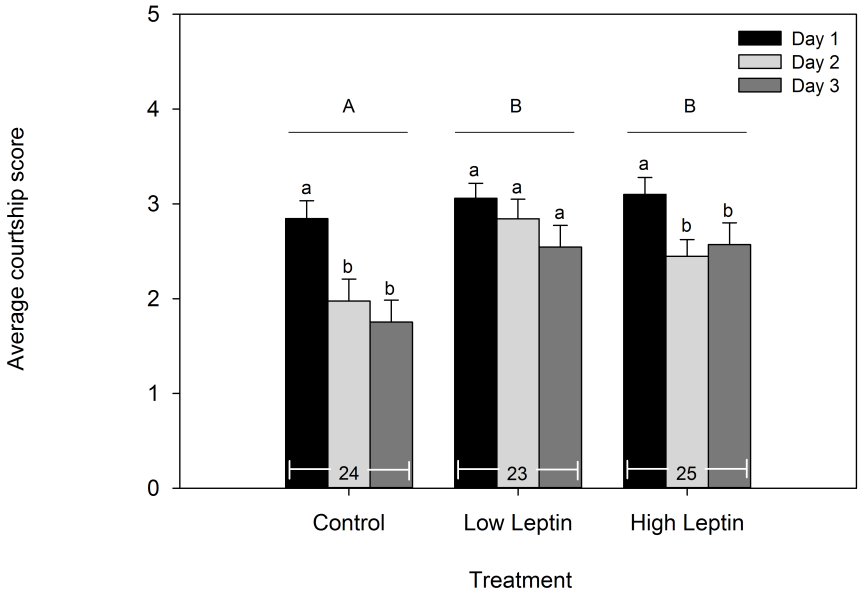

Figure 3.3. The effect of exogenous leptin on average male courtship score in red-sided garter snakes. Snakes were injected with 0, 3, or 30 µg of mouse recombinant leptin (Control, Low leptin, or High leptin, respectively) for 3 days. Males were subjected to a mating trial on each injection day for a total of three mating trials. Numbers along the x-axis represent sample sizes in each group. Statistically significant differences among treatment groups are indicated by capital letters above the horizontal lines. Lower case letters indicate significant differences among days within each treatment group.

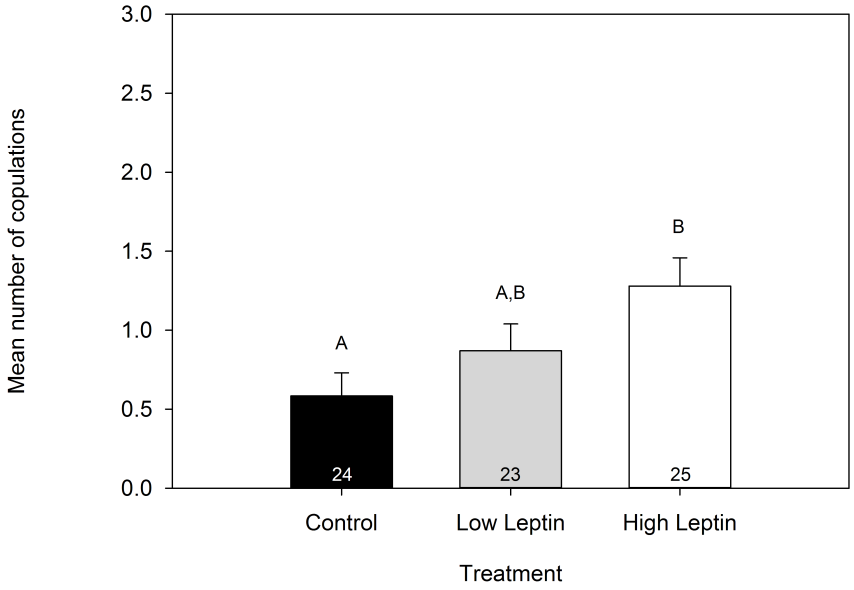

Figure 3.4. The effect of exogenous leptin on the number of copulations a male red-sided garter snake achieved. Males were injected with 0, 3, or 30 µg of mouse recombinant leptin (Control, Low leptin, or High leptin, respectively) for 3 days. Males were subjected to a mating trial on each injection day; we limited males to one copulation per day. Numbers along the x-axis indicate sample sizes. Letters indicate statistically significant differences among treatment groups.

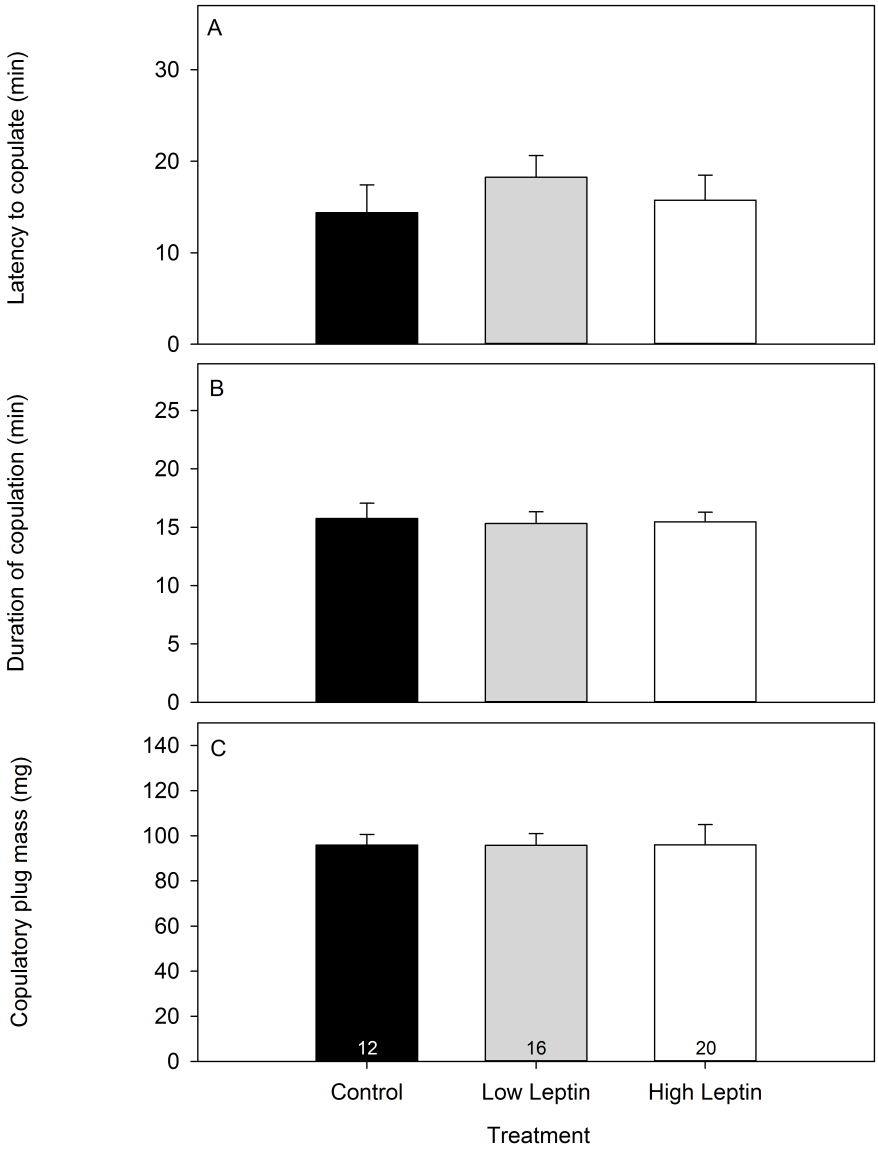

Figure 3.5. The effect of exogenous leptin on (A) latency to copulate, (B) duration of copulation, and (C) copulatory plug mass in male red-sided garter snakes. Snakes were injected with 0, 3, or 30 µg of mouse recombinant leptin for 3 days (Control, Low, or High leptin treatment groups, respectively). Males were subjected to a mating trial on each injection day; data (mean + 1 s.e.m.) presented here include only the first mating of those males that copulated regardless of what day the mating occurred on. Numbers along the x-axis in (C) indicate sample sizes for each treatment group in all panels.

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Chapter 4

The preferential use of energy stores during hibernation depends on temperature and sex in red-sided garter snakes

Abstract

Many organisms hibernate to avoid harsh winter conditions and delay the depletion of energy stores until suitable conditions return. Investigations into physiological responses to hibernation heavily focus on mammals and point to the preferential use of adipose stores. Few studies examine how different temperature influences these processes, with fewer studies investigating ectothermic animals. Further, investigating organisms that reside at higher latitudes are of particular interest because climate change disproportionally affects these regions. We investigated the utilization of energy substrates during hibernation in red-sided garter snake (*Thamnophis sirtalis parietalis*), one of the most northerly-residing populations of snakes. Snakes were subjected to simulated hibernation at either 4 or 12°C and sampled throughout a 16 week period to measure adipocyte follicle size and liver glycogen. Our data suggest that red-sided garter snakes preferentially utilize liver glycogen during hibernation, as it significantly decreased while adipocyte follicle size did not. These findings conflict with the mammalian literature. While liver glycogen decreased over hibernation, we found no effect of hibernation temperature on female energy stores. Conversely, energy stores were depleted to a greater

extent in males hibernated at 12°C compared to 4°C. Because energy stores facilitate reproductive behavior in many organisms, including red-sided garter snakes, warmer hibernation temperatures will decrease the available energy for mating activities in males upon spring emergence. With the increasing global temperatures caused by climate change, this research provides insight as to how increased temperatures influence organismal physiology and behavior, a link that may profoundly and negatively affect an organism's Darwinian fitness.

Introduction

Seasonal fluctuations in weather often expose animals to prolonged periods of unsuitable conditions such as extreme aridity or cold. In response to such extreme environmental conditions that can result in reduced food availability, animals enter a period of dormancy (e.g., estivation or hibernation) that coincides with a decrease in activity and metabolism, allowing organisms to delay the depletion of energy stores. Investigations of the physiological responses to hibernation focus heavily on mammals, ranging from rodents to bears, and points to the preferential usage of adipose stores to fuel physiological processes during hibernation (Dark, 2005; Farley and Robbins, 1995; Galster and Morrison, 1976; MacCannell et al., 2019; Shimozuru et al., 2016; Siutz et al., 2017). The preferential use of one energy substrate over another during low-temperature dormancy has received far less attention in ectothermic animals.

Unlike the mammalian literature, evidence suggests that reptiles may utilize liver glycogen stores over adipose stores for energetic demands during

hibernation (lizards: Barwick and Bryant, 1966; Naya et al., 2008; Oliveira et al., 2018; but see Souza et al., 2004; turtles: Crawford, 1994; snakes: Aleksiuk and Stewart, 1971; Costanzo, 1985). Whether other terrestrial ectotherms preferentially utilize liver glycogen stores over fat stores is equivocal because few studies have investigated changes in energy substrate in response to hibernation. In an Alaskan population of wood frogs (*Rana sylvatica*), fat body weight significantly decreased between pre-hibernatory and hibernatory individuals, while liver glycogen significantly increased (Costanzo et al., 2013). However, this increase in liver glycogen is likely an adaptation specific to wood frogs to increase osmolarity of tissues for freeze tolerance. In Cunningham's spiny-tailed skink (*Egernia cunninghami*), liver glycogen decreased during the hibernation period, whereas no clear pattern of fat body weight was observed (Barwick and Bryant, 1966). Liver masses of the Andean lizard (*Liolamemus bellii*) were significantly higher prior to hibernation compared to afterwards, while fat body weight did not decrease between these time points (Naya et al., 2008). Post-hiberatory painted turtles (*Chrysemys picta*) display greater decreases in total body glycogen than total lipid content compared to pre-hibernatory individuals (Crawford, 1994).

Because snakes exhibit a wide range of life-history traits (e.g., prey acquisition techniques, oviparity versus viviparity), snakes provide a unique opportunity to explore to what extent traits may be taxonomically constrained or linked to an animal's ecology. Eastern garter snakes (*Thamnophis sirtalis sirtalis*) sampled just prior to entering hibernation had significantly higher liver glycogen

compared to snakes at emergence, but over the same period fat stores did not change (Costanzo, 1985). The total lipid content and liver weight of red-sided garter snakes (*Thamnophis sirtalis parietalis*) are lower upon emergence from hibernation compared to individuals sampled in the fall prior to hibernation, while total body protein content does not fluctuate (Aleksiuk and Stewart, 1971). Along with these two garter snake studies, the majority of studies on reptiles infer the usage of energy stores during hibernation by examining the time points immediately before and after this life-history stage. Directly sampling individuals during hibernation would unequivocally determine which energy stores contribute to fueling biological processes.

In light of the current and projected effects of climate change, many animals will continue to be exposed to conditions outside of their historical temperature range. Upon exposure to temperatures outside of normal conditions during an organism's active period, physiological processes such as growth (Warner et al., 2020; Zhang et al., 2020; but see Romero-Diaz et al., 2017) and reproduction (Pankhurst and Munday, 2011; Willette et al., 2005; Zhang et al., 2020) can be adversely affected. Research investigating If and how animals alter physiology in response to changing hibernation temperatures has received little attention. During simulated hibernation, red-sided garter snakes exposed to a warm-temperature treatment exhibited lower plasma androgen levels compared to a cold-temperature treatment (Lutterschmidt and Mason, 2009). However, research investigating the physiological responses to differing hibernation temperatures are absent as it pertains to energy metrics in ectotherms.

Therefore, we aimed to determine how stored forms of energy change during hibernation in red-sided garter snakes, and also if varying hibernation temperatures differentially affect the use of those energy stores.

Red-sided garter snakes are a valuable model for investigating the effects of hibernation temperatures on physiology. First, the population of garter snakes in Manitoba, Canada is one of the most northerly residing reptiles. Higher latitudes are projected to have greater increases in temperature (IPCC, 2013). Therefore, investigating responses of organisms to elevated temperatures that inhabit higher latitudes will provide insight into how these animals may be affected. Second, because the expansive range of the common garter snake (*T. sirtalis*) extends from southern Canada to the Gulf of Mexico, we can examine how an animal of a single species respond physiologically to varying temperatures across a range of latitudes. Lastly, northern populations of common garter snakes rely solely on stored forms of energy to fuel physiological processes during hibernation. Red-sided garter snakes are not only aphagic throughout winter dormancy but also through the spring mating season (Gregory, 1977; Gregory and Stewart, 1975). As the mating season progresses, snakes migrate up to 17 km to summer feeding grounds. After spending the summer feeding, snakes migrate back to overwintering sites and once again become aphagic in the fall. Thus, the long period of aphagia (i.e., approximately 9 months) exhibited by red-sided garter snakes allows us to determine how energy substrates contribute to fueling energy demands during hibernation in the absence of intermittent feeding. For these reasons, we investigated how body

condition index, adipocyte follicle size, and liver glycogen vary in male and female red-sided garter snakes throughout hibernation. We also examined how different hibernation temperatures affected these metrics. We predicted that garter snakes preferentially utilize liver glycogen to a greater extent than adipose stores during hibernation, and elevated hibernation temperatures would deplete energy stores to a greater degree than low hibernation temperatures.

Materials and methods

Animal collection and care

Snakes (females: n = 40; males: n = 84) were collected in Inwood, Manitoba, Canada from 16-18 September 2016 and transported to Portland State University, arriving on 21 September. The Portland State University Institutional Animal Care and Use Committee (protocol number 25) and Manitoba Department of Sustainable Development (collecting permit WB18801) approved all experimental procedures. Snakes were acclimated in environmental chambers to a photoperiod of 11:13 h L:D and thermoperiod of 18:12°C for 4 weeks. They were provided with water ad libitum, but were not offered food as snakes are aphagic during this time. On 20 October, snakes were subjected to simulated hibernation in complete darkness at either 4°C or 12°C. We measured mass and snout-vent length (SVL) to ensure initial morphometrics did not differ between temperature groups or among sampling time points during hibernation. Additionally, we measured mass at the time of euthanasia to determine if body condition index differed with hibernation temperature or duration. We determined

body condition index separately for each sex because female red-sided garter snakes are larger and heavier than male snakes. Body condition index was determined by calculating the residual from a regression of log-transformed body mass on log-transformed SVL.

Tissue collection

We collected adipose and liver tissues from male snakes after 0, 4, 8, and 16 weeks into hibernation (n = 6, 12, 12, and 12, respectively, for each hibernation temperature). We collected tissues from females after 0, 8, and 16 weeks of hibernation (n = 4, 8, and 8, respectively, for each hibernation temperature). We reduced sample sizes at the week 0 time point because this sampling point was prior to snakes entering hibernation and the manipulation of temperature. We used this sampling point to test for baseline differences between snakes housed in the two environmental chambers, and therefore we did not expect to see differences in initial metrics. We chose three sampling time points for females to decrease sample sizes based on preliminary results from our lab (Lutterschmidt and Main, 2015). Snakes were euthanized for collection of tissues by injection of an overdose of sodium Brevital near the heart. We excised adipose and liver tissues to measure adipocyte follicle area using histology and liver glycogen using an enzymatic assay. Brains were also collected for a separate experiment that is not part of the analyses presented here. Adipose tissue was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer overnight and then stored in 0.1 M phosphate buffer (pH 7.2) at 4°C until we processed

samples for histology. Liver samples were flash frozen on dry ice and stored at -80°C until we performed glycogen assays.

Adipose tissue histology

Tissue processing, embedding, and sectioning followed procedures as described in Wilson and Lutterschmidt (*in press*). Briefly, tissues were dehydrated in increasing concentrations of ethanol, cleared in Citrisolv, and then impregnated with paraffin overnight. Specimens were sliced at 5 μm using a microtome and collected onto gelatin-coated microscope slides. After collection, sections were deparaffinized for 10 min in Citrisolv and then rehydrated in decreasing concentrations of ethanol, followed by counterstaining with hematoxylin and eosin. Adipocyte follicle area was measured using the Image J software Adiposoft (National Institutes of Health, Bethesda, MD, USA). When possible, we measured 25 follicles per section of adipose tissue in four different sections per individual for a total of 100 follicles for each snake. The mean number of follicles (± s.e.m.) measured was 82 ± 3.6 for females and 93 ± 1.6 for males. Individuals with less than 25 measurable follicles were excluded from analysis (females: n = 3; males: n = 7). Due to a high tissue to fixative ratio, these individuals' samples were under-fixed and we could not process them.

Glycogen enzymatic assay

Similar to procedures described by Wilson and Lutterschmidt (*in press*), we homogenized 100-120 mg of liver tissue in 0.5 mL of 1% HALT protease inhibitor (product 1862209, Thermo Scientific, Waltham, MA, USA) in assay

buffer (0.936% Na_2PO_4, 0.4656% KPO_4, 1% NaCl). One individual had only 89 mg of liver tissue collected, but we kept that individual in the analysis as liver glycogen is measured relative to the amount of tissue homogenized. However, two males from the week 8 time point in the 12°C temperature treatment had less than 50 mg of tissue collected. We did not process these samples because homogenization protocols are often based on ratios of tissue weight to volume of homogenization solution and we thought that less than 50% of the ideal tissue weight could provide erroneous results. Liver homogenates were centrifuged at 800 g for 10 minutes at 4°C. After transferring supernatant to a fresh tube, we diluted it to a ratio of 1:4 in assay buffer and prepared each sample in duplicate for analysis. We digested glycogen to glucose by adding 25 µL of glycogen hydrolysis enzyme reconstituted in 3 mL of 50 mM sodium acetate (Item no. 700483, Cayman Chemical, Ann Arbor, Michigan, USA) to 5 µL of diluted supernatant. To measure background glucose levels in each sample, we incubated 5 µL of diluted supernatant with 25 µL of 50 mM sodium acetate (i.e., without enzyme). All samples were incubated at 37°C for 30 min. We measured the glucose concentration of each sample using a Contour Next EZ glucose meter (Ascensia Diabetes Care, Parsippany, New Jersey, USA). Because all background glucose levels were below the level of detection (10 mg dL^{-1}), we did not subtract background glucose from glycogen measurements. Prior to using the glucose meter to measure these samples, we ensured that measurements between the glucose meter and the colorimetric assay used in Wilson and Lutterschmidt (*in press*; Item no. 700483, Caymen Chemical, Ann Arbor,

Michigan, USA) significantly related to each other ($R^2 = 0.96$, $p < 0.001$; Figure 4.1). Twenty-three individuals were run per assay; samples were randomly distributed across a total of six assays. We ran the same standard in duplicate at the beginning and end of each assay to measure intra- and inter-assay variation (8.56 ± 1.42 and 8.16, respectively).

Statistical analyses

To adhere to the assumptions of parametric statistical tests, we either square root- or log- transformed data where necessary. If data could not be transformed to meet the assumptions of statistical tests, we used non-parametric tests. All statistics were run in Sigma Stat 12.5 (Systat Software, Inc). We ran separate statistical analyses for each sex for two reasons: (1) due to an unbalanced study design in which a week 4 sampling time point for females was not collected; and (2) because female red-sided garter snakes exhibit a larger adipocyte follicle area, even after controlling for SVL, and higher liver glycogen content compared to males (Wilson and Lutterschmidt, *in press*). We used two-way analysis of variance (ANOVA) to ensure that initial morphometrics (e.g., mass, SVL, and body condition index) did not differ among sampling times or between hibernation temperatures in male and female snakes. To examine the effect of hibernation temperature and duration on energy metrics, we also used two-way ANOVAs. To further examine significant main factors, we ran Tukey's post hoc tests.

Results

Morphometrics

All initial body measurements (i.e., mass, SVL, and body condition index) in male and female red-sided garter snakes did not differ among sampling times or between hibernation temperatures. Male body condition index was not influenced by hibernation duration (Figure 4.2A; $F_{3,76} = 1.46$, $p = 0.232$), hibernation temperature ($F_{1,76} = 1.99$, $p = 0.162$), or the interaction between hibernation duration and temperature ($F_{3,76} = 1.12$ $p = 0.347$). Body condition index decreased over hibernation in females (Figure 4.2B; $H = 5.98$, $p = 0.050$) but was not influenced by hibernation temperature ($H = 0.12$, $p = 0.724$) or the interaction between hibernation duration and temperature ($H = 1.95$, $p = 0.376$). Post-hoc tests revealed that body condition index was significantly lower in females after 16 weeks of hibernation compared to individuals at week 0 (Figure 4.2B).

Adipocyte follicle area

For male snakes, neither duration of hibernation (Figure 4.3A; $F_{3,69} = 0.96$, $p = 0.419$) nor hibernation temperature ($F_{1,69} = 2.21$, $p = 0.142$) affected adipocyte follicle size, but there was a significant interaction between hibernation duration and temperature ($F_{3,69} = 3.65$, $p = 0.017$), indicating that the changes observed in male adipocyte follicle size during hibernation depended on temperature. Post-hoc tests revealed that adipocyte follicle size decreased over hibernation in males hibernated at 12°C, whereas adipocyte follicle size did not change in males hibernated at 4°C (Figure 4.3A). Despite no differences in initial

mass, SVL, or body condition index in males at week 0, we found that adipocyte follicle size of males in the 12°C temperature group was significantly higher at week 0 than that of males in the 4°C temperature group. In females, adipocyte follicle size was not influenced by hibernation duration (Figure 4.3B; $F_{2,31} = 0.88$, $p = 0.426$), hibernation temperature ($F_{1,31} = 1.85$, $p = 0.183$), or the interaction between these factors ($F_{2,31} = 0.41$, $p = 0.960$). Correcting adipocyte follicle size for SVL did not change these relationships in either male or female snakes.

Glycogen content

Liver glycogen of male snakes decreased over hibernation (Figure 4.4A; $F_{3,69} = 17.20$, $p < 0.001$) but did not differ with the main effects of hibernation temperature ($F_{1,69} = 0.22$, $p = 0.639$). However, there was a significant interaction between hibernation duration and temperature on male liver glycogen (Figure 4.4A; $F_{3,69} = 7.18$, $p < 0.001$), with males hibernated at 12°C displaying a greater decrease in liver glycogen after 16 weeks compared to males hibernated at 4°C. In females, liver glycogen decreased with hibernation duration (Figure 4.4B; $F_{2,34} = 14.82$, $p < 0.001$) but did not differ with hibernation temperature ($F_{1,34} = 0.67$, $p = 0.419$). The interaction between hibernation duration and hibernation temperature was not significant ($F_{2,34} = 1.26$, $p = 0.297$). Post-hoc tests revealed that liver glycogen significantly and progressively decreased over hibernation (Figure 4.4B).

Discussion

We present several changes in energy metrics of red-sided garter snakes over hibernation. In males, although body condition index did not significantly change during hibernation, both adipose stores and liver glycogen decreased. These changes depended upon hibernation temperature in male snakes, with a higher hibernation temperature depleting energy stores to a greater extent. Females displayed different patterns of change over hibernation. Body condition index and liver glycogen of female snakes significantly decreased during hibernation, but we found no evidence to suggest that adipose stores were utilized. Further, females did not display any temperature-dependent differences in energy metrics during hibernation, unlike male snakes. One consistent finding between the sexes was the utilization of liver glycogen during hibernation, as liver glycogen of both sexes significant decreased over the 16-week dormancy period.

Our findings suggest that garter snakes preferentially utilized liver glycogen stores during low-temperature dormancy because our findings showed that liver glycogen significantly decreased during a 4°C simulated hibernation in both sexes, whereas adipocyte follicle size did not. These results align with previous research in eastern garter snakes that showed liver glycogen, but not fat body mass, was significantly lower at emergence compared to the onset of hibernation (Costanzo, 1985). The preferential use of liver glycogen stores contrast with previous predictions in red-sided garter snakes positing that lipid and protein substrates are important during times of aphagia (Aleksiuk and Stewart, 1971). Although not investigated in this study, the utilization of proteins

to fuel physiological processes during hibernation in garter snakes cannot be discounted (Costanzo, 1985).

Similar to our findings, some evidence in lizards suggests that reptiles preferentially utilize stored energy in the liver to meet the energetic demands of hibernation. For instance, liver glycogen of Cunnighham's spiny-tailed skinks (*Egernia cunninghami*) significantly decreased over hibernation, but total fat body mass did change significantly (Barwick and Bryant, 1966). Liver mass, but not dry fat body mass, was significantly higher prior to entering winter hibernation than at emergence in Andean lizards (*Liolaemus bellii*; Naya et al., 2008). Lastly, liver glycogen stores in male, but not female *Tropidurus catalanensis* (no common name) significantly decreased from winter to spring (Oliveira et al., 2018). However, juvenile tegu lizards (*Tupinambis merianae*) utilize both liver glycogen and abdominal fat stores during hibernation (Souza et al., 2004). Further investigations into stored energy metrics of additional reptilian species would help clarify if the preferential usage of liver glycogen depends on sex and/or ontogeny.

We found no evidence to suggest that garter snakes utilized adipose stores during the ecologically-relevant hibernation condition of 4°C. These results differ from numerous mammalian species displaying a decrease in adipose tissue metrics over hibernation, thereby contributing to the theory that mammals utilize adipose tissue as the primary source of energy to fuel biological processes during hibernation (Dark, 2005; Farley and Robbins, 1995; Galster and Morrison, 1976; MacCannell et al., 2019; Shimozuru et al., 2016; Siutz et al., 2017). We

acknowledge that measuring adipose-related energy stores through histological measurements such as adipocyte follicle area in this study or total fat body mass in other studies (Aleksiuk and Stewart, 1971; Costanzo, 1985) may not provide enough acuity in detecting changes in fatty acid metabolism. However, the finding that adipocyte follicle area significantly decreased in male garter snakes hibernated at 12°C suggests examining adipocyte follicle area can detect changes in stored triglyceride catabolism. Regardless, investigating changes in energy stores at the substrate level (i.e., fatty acids) would clarify if garter snakes also utilize fat stores to meet energetic demands during hibernation.

In preparation for hibernation, mammalian species deposit substantial fat stores during a period of hyperphagia (Fietz et al., 2003; Kronfeld-Schor et al., 2000; Sheriff et al., 2013). Our data, in conjunction with the findings of Wilson and Lutterschmidt (*in press*), may point to red-sided garter snakes depositing substantial fat stores in preparation for hibernation. During the spring, male red-sided garter snakes had a mean adipocyte follicle area of 250 μm^2, whereas male garter snakes sampled just prior to simulated hibernation in this study had a mean adipocyte follicle area of 463 μm^2 ± 35. A near doubling of adipocyte follicle size points to substantial fat deposition prior to hibernation, a finding that is similar to mammals but contrary to a previous suggestion in garter snakes by Aleksiuk and Stewart (1971). Although measuring glucose levels with the meter reports concentrations approximately one in half times greater than the colorimetric assay, this discrepancy doesn't account for the more than doubling of liver glycogen levels in the fall (Females: 0.83 mg/dl ± 0.06; Males: 0.78 ±

0.06) compared to the spring (0.30 and 0.24 mg/dl for females and males, respectively). This suggests that both forms of energy stores in red-sided garter snakes are substantially higher in the fall compared to the spring.

In comparing the suggestion that reptiles preferentially utilize glycogen stores to the more established theory that mammals preferentially utilize fat stores, the question becomes “Why would different animals utilize different energy substrates to fuel physiological processes during hibernation?” Perhaps hibernation is not as energetically demanding in reptiles as it is in mammals. During hibernation, mammals perform periodic bouts of arousal from torpor where individuals increase metabolism and body temperature. The timing of these torpor bouts generally fall into one of two categories: approximately every 24 hours or between 2-32 days depending on the species (Ruf and Geiser, 2015). This is in contrast to hibernating ectotherms where body temperature remains relatively stable and reflects ambient temperature (Barwick and Bryant, 1966; Brown, 1982; Etheridge et al., 1983; Macartney et al., 1989). For instance, the body temperature of red-sided garter snakes from field measurements parallels ground temperature, gradually decreasing from 14.7 to 1.1°C over the course of winter dormancy (Lutterschmidt et al., 2006). Therefore, it is likely that the difference in thermal biology between endotherms and ectotherms results in a higher energetic demand for endotherms during hibernation.

Additional physiological responses likely contribute to higher energetic costs for endothermic hibernators. In response to quickly upregulating and downregulating metabolism, mammals initiate the cellular stress response and

increase antioxidant proteins during hibernation, which likely protect against cellular damage (Carey et al., 2003; Grabek et al., 2015; Yin et al., 2016). For example, mammals significantly increase their expression of Nrf2, a transcription factor for antioxidant proteins, in response to hibernation (Storey et al., 2010). Few studies have investigated how hibernation influences gene expression associated with the cellular stress response in reptiles: juvenile Chinese soft-shelled turtles (*Pelodiscus sinensis*) increase expression of Nrf2 in liver and heart tissues during hibernation, although the change was statistically nonsignificant (Zhang et al., 2017). Hibernating Australian central bearded dragons (*Pogona vitticeps*) increase more than 150 genes associated with the cellular stress response (Capraro et al., 2019). In relating their findings to various other mammalian species and one species of frog, Capraro et al. (2019) found no consistent pattern in the cellular stress response to hibernation in either ectotherms or endotherms at the transcriptomic level. A direct comparison of the genes and/or proteins associated with the cellular stress response in ectotherms and endotherms would inform the cost of hibernation and may provide insight into the likely taxonomic differences in the preferential usage of energy stores.

Although not explicitly tested here, we suggest that red-sided garter snakes exhibit a few sexually dimorphic differences in energy metrics during hibernation. First, body condition index decreases in female snakes, but does not change significantly in males throughout hibernation. What information body condition index conveys as it relates to stored forms of energy remains unknown in red-sided garter snakes. Despite significant changes in body condition index

during hibernation, adipocyte follicle area did not change significantly in female snakes, indicating that body condition index was not directly related to adipocyte follicle area. Similarly, during the spring mating season, body condition index is not significantly related to either adipocyte follicle area or liver glycogen in garter snakes (Wilson and Lutterschmidt, *in press*). A closer examination of male red-sided garter snakes in this study further highlights the uncertainty of how body condition index relates to stored energy. Prior to subjecting males to the different temperature treatments, we found that male snakes in the 4°C temperature group had significantly smaller adipocyte follicles compared to males in the 12°C temperature group, despite having no significant differences in initial SVL, body mass, or body condition index. Because different metabolic components (e.g., mass of muscle, skeleton, or fat) significantly contribute to body condition in different snakes (Shine and Mason, 2005; Waye and Mason, 2008), species-specific validations may illuminate how body composition is shaped by ecological factors. Further, because the physiology and behavior of red-sided garter snakes varies with body condition (e.g., Dayger et al., 2013), identifying which body metrics contribute to body condition would help elucidate how metabolic substrates differentially influence behavior and physiology.

Similar to Costanzo (1985) and Wilson and Lutterschmidt (*in press*), male red-sided garter snakes had substantially smaller adipocyte follicles than females. Further highlighting sexually driven differences in energy usage, we also provide evidence to suggest that male red-sided garter snakes were more sensitive to temperature changes over hibernation than females. Males

hibernated at 12°C displayed greater decreases in both adipocyte follicle size and liver glycogen compared to males hibernated at 4°C, suggesting that warmer hibernation temperatures were more energetically costly for male garter snakes. In contrast, females do not incur a higher energetic cost in warmer hibernation temperatures as evidenced by the lack of significant differences in body condition index, adipocyte follicle size, or liver glycogen between temperature treatments. Consistent with hibernating eastern garter snakes, we found no evidence to suggest that hibernating red-sided garter snakes exhibited a sexual dimorphism in liver glycogen consumption, as liver glycogen decreased significantly in both male and female snakes in both hibernation groups. Lower energy stores at the time of emergence likely result in decreased reproductive effort during the spring mating season for male garter snakes, as substantial evidence suggests that reproduction and stored forms of energy positively relate (Alonso-Fernández and Saborido-Rey, 2012; Barron and Andraso, 2001; Becker et al., 2013; Bronson and Marsteller, 1985; Dayger et al., 2013; Doughty and Shine, 1997; Doughty and Shine, 1998; Groscolas et al., 2008; Kirk, 1997; Long, 1987; Smith and Moore, 2003). In light of climate change, the sensitivity of male snakes to increased hibernation temperatures may mean that males have lower amounts of available stored energy upon emergence. Therefore, we provide a potential metabolic link between hibernation temperature, reproduction and fitness. To what extent decreased energy stores during the spring mating season affect fitness remains unclear in red-sided garter snakes, because energy stores do not appear to influence the decision to migrate from the breeding grounds to summer

feeding areas in search of feeding (i.e., self-maintenance) opportunities (Wilson and Lutterschmidt, *in press*).

In conclusion, our findings suggest multiple sex differences in energy metrics during hibernation: body condition index decreased in females, but we detected no significant changes in male body condition over hibernation. Additionally, males were more sensitive than females to elevated hibernation temperatures as it pertains to adipose and liver glycogen stores. We also suggest that garter snakes preferentially utilize liver glycogen over adipose stores to fuel physiological processes during hibernation, a finding that is consistent with lizards but contrary to mammals. These snakes have only three months to accumulate enough energy stores to survive hibernation and also engage in an intense mating period upon emergence. Higher energetic demands during hibernation due to elevated temperatures as a result of climate change could have detrimental effects on the allocation of energy reserves for reproductive processes. Increasing temperatures may further compromise red-sided garter snake fitness as evidence suggests climate change decreased activity times in various *Sceloporus* species (Sinervo et al., 2010). Without interventions to assuage the effects of climate change, we may observe a substantial decrease in the fitness of these and other hibernating ectotherms. Because red-sided garter snakes, like many animals, are secondary consumers (Hart, 1975), the resulting population declines could in turn have broad, negative impacts on the greater biological community.

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Figures

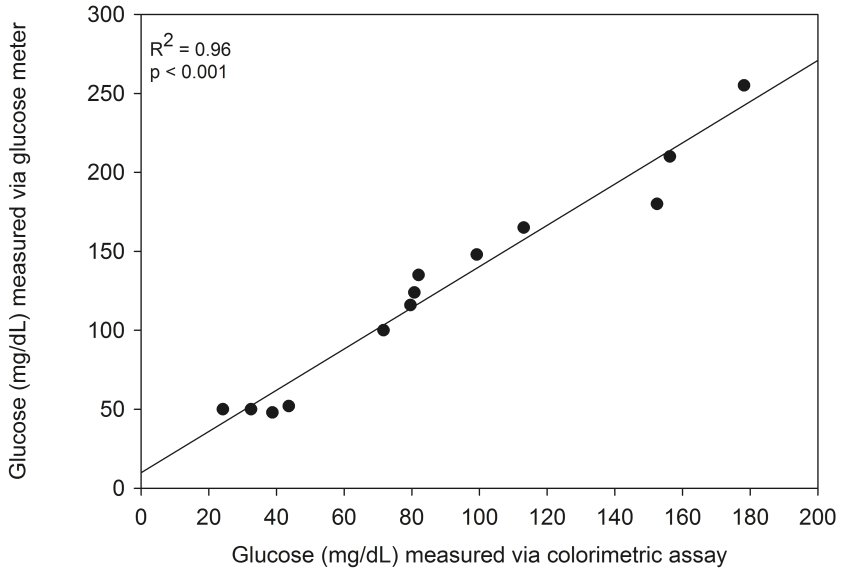

Figure 4.1. Comparison of methods to quantify liver glycogen in red-sided garter snakes (*Thamnophis sirtalis parietalis*). Glycogen was digested to glucose in supernatant of liver homogenates by exposure to amylogucosidase. Glucose values measured by a glucose meter significantly related to the values of the same samples measured by a colorimetric assay.

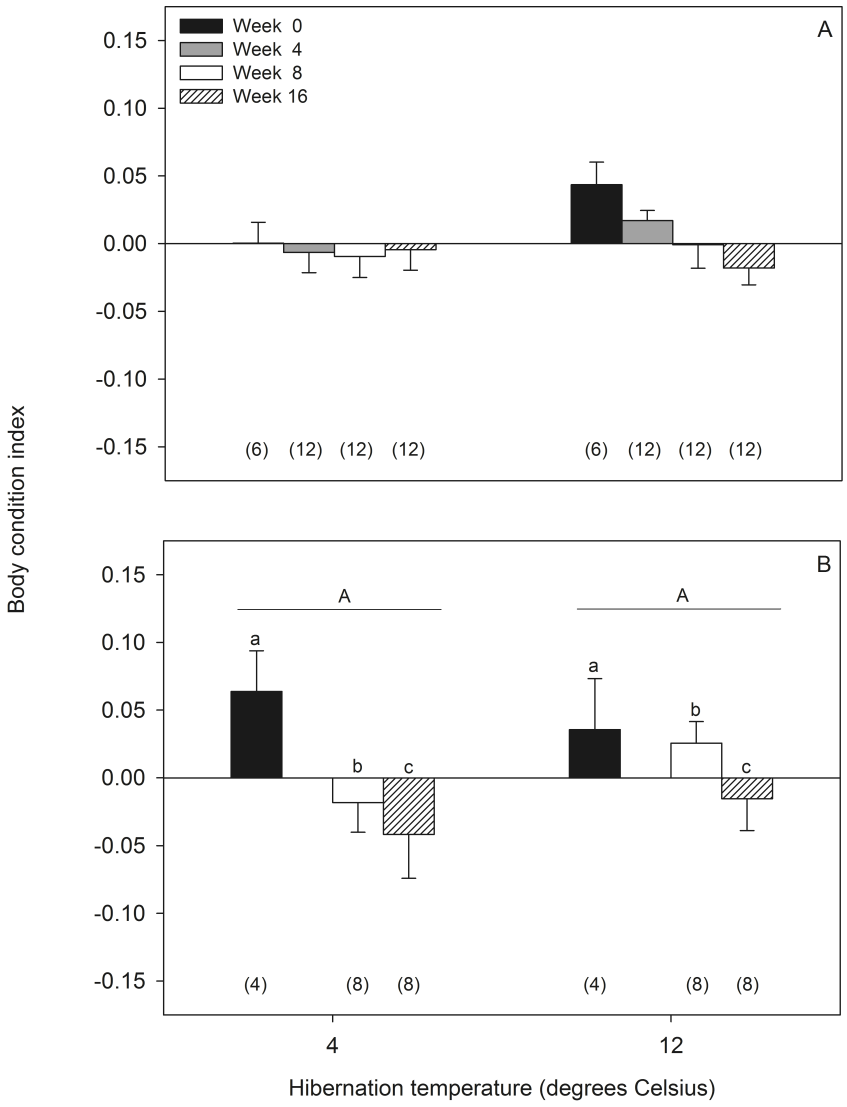

Figure 4.2. The effects of hibernation temperature and duration on body condition index of male (A) and female (B) red-sided garter snakes (*Thamnophis sirtalis parietalis*). Body condition index was determined by calculating the residual from a regression of log-transformed body mass on log-transformed SVL separately for male and female snakes. In female snakes, the main factor of hibernation duration was statistically significant and different lower case letters indicate significant differences across time points. All data are the mean + or - 1 s.e.m. Numbers in parentheses along the x-axes indicate sample sizes at each time point.

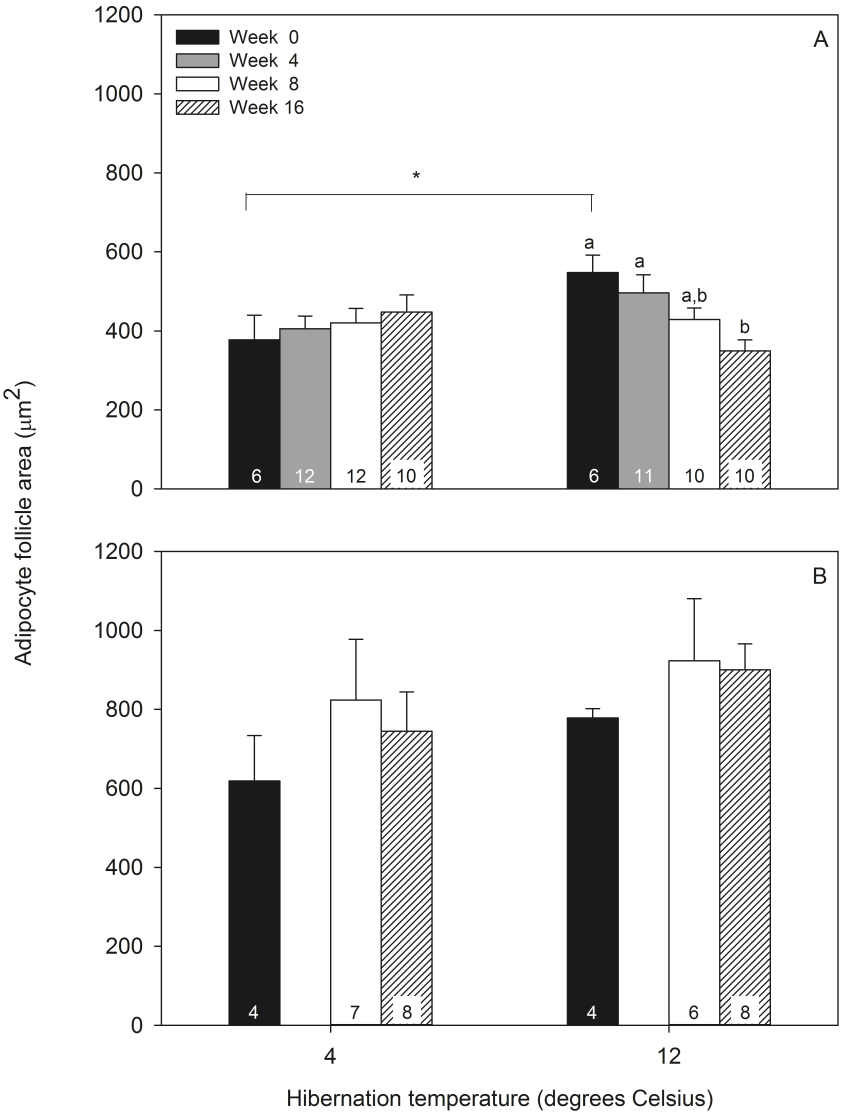

Figure 4.3. The effects of hibernation temperature and duration on adipocyte follicle size in male (A) and female (B) red-sided garter snakes (*Thamnophis sirtalis parietalis*). In male snakes, there was a significant interaction between hibernation temperature and duration on adipocyte follicle size. Lower-case letters indicate significant differences across sampling times at 12°C, while the asterisk indicates an initial significant difference between males in the 4 and 12°C temperature groups. All data are the mean + 1 s.e.m. Numbers along the x-axes indicate sample sizes at each time point.

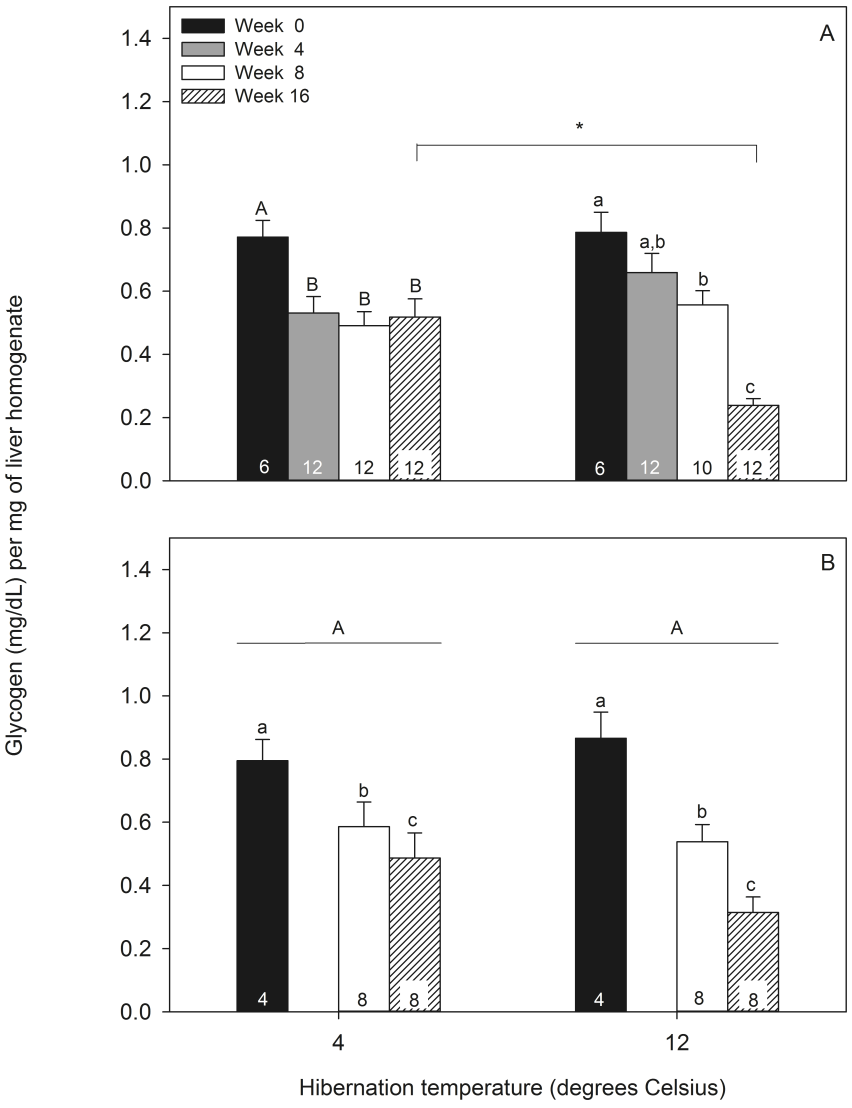

Figure 4.4. The effects of hibernation temperature and duration on liver glycogen in male (A) and female (B) red-sided garter snakes (*Thamnophis sirtalis parietalis*). In male snakes, we found significant effects of hibernation duration and the interaction of hibernation temperature and duration. While male snakes hibernated at both 4 and 12°C display a decrease in liver glycogen content over hibernation, 12°C males sampled after 16 weeks of hibernation display a significantly greater decrease in liver glycogen compared to 4°C males (indicated by the asterisk). Capital letters indicate significant differences across males hibernated at 4°C, whereas lower-case letters indicate significant differences across sampling times in males hibernated at 12°C. In female snakes, there was a significant main effect of hibernation duration on liver glycogen; lower-case letters indicate significant differences across time points.

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Chapter 5

Discussion and Conclusions

My doctoral research aimed to investigate how energy metrics influence life-history stages and transitions surrounding reproduction by measuring adipocyte follicle size and liver glycogen concentrations. As such, this is the first comprehensive investigation that determined if multiple forms of stored energy relate to life-history stages in a reptilian species. Expanding research to include comparative models provides insight into the spectrum of physiological processes that occur across taxa and allows a view into how those mechanisms evolve given enough time and pressure. Further, understanding when organisms prioritize the usage of certain energy stores over others could assist in better management and conservation practices, especially in light of climate change.

By probing different aspects of the annual life-history cycle in red-sided garter snakes during which time they are aphagic, I aimed to determine how stored forms of energy influenced life-history stages and transitions. In Chapter 2, I asked if energy substrates differ with sex and migratory status to determine if adipose and glycogen stores influence when red-sided garter snakes decide to migrate. I then looked within the spring mating season to determine how energy stores, particularly those stored in adipose tissues, influence reproductive behavior. And lastly, I wanted to determine how energy stores change leading up to the spring mating season during hibernation.

Sex differences

A common theme prevalent throughout my dissertation is the sex-specific differences in energy substrates exhibited by red-sided garters snakes. During the spring mating season, females store more energy in the form of adipose tissue and liver glycogen than male snakes. This relationship persisted even after correcting for snake size because females are heavier and longer than males suggesting that females store more fatty acids than size-matched males. In changing a snake's perceived amount of adipose stores, I also found sex-specific responses in reproductive behavior. While leptin promoted appetitive (i.e., courtship behavior) and consummatory (i.e., the number of copulations) sex behavior in male snakes, I only provide evidence to suggest that leptin promotes consummatory sex behavior in females. And lastly, in addition to females storing more fat than males, I observed multiple sexual dimorphisms in energy status during hibernation. Females also displayed a significant decrease in body condition index during hibernation, but unlike males, were insensitive to hibernation temperature effects on energy status.

These lines of evidence strongly suggest that females and males differ in their use of energy substrates. As discussed in Chapter 2 (page 44), snakes utilizing different reproductive tactics display differences in various morphometrics including stored forms of energy (Bonnet et al. 1998). These sexually dimorphic differences in energy status likely arise from sexual conflict, a robust area of research in red-sided garter snakes (Shine et al. 2000, 2001, 2004, 2005; Friesen et al. 2013, 2014a, 2014b). Females have limited ability to

exert mate choice at the den due a functionally skewed sex ratio (Shine et al. 2000). However, when a female migrates away from the den, mating ball sizes are substantially smaller, likely allowing her more choice in copulations (Shine et al. 2001). Some evidence suggests that smaller females are disproportionally impeded from migrating away from the den as males court and attempt to copulate with them (Shine et al. 2000; Wilson and Lutterschmidt, personal observation; but see Shine et al. 2004). If it is easier for larger females to migrate away from the den, and by doing so allowing her more control in mate choice, females may prioritize growth over other processes. Additionally, larger female garter snakes produce more and/or heavier offspring per reproductive bout (Gregory 1977; Ford and Karges 1987; Dunlap and Lang 1990; Gregory 2006; Wilson and Lutterschmidt unpublished data). Such selection pressures may help explain why females preferentially store energy substrates, especially in light of the short time frame that theses snakes are able to obtain nutrients and energy during the summer before migrating back to overwintering sites. Because females are thought to be biennial breeders, it is likely that energy stores obtained in prior summers influence the ability of a female to both reproduce and/or grow in subsequent summers. Indeed, evidence suggests female red-sided garter snakes are capital breeders (Gregory 2006). Studies employing radiolabeled carbon during the feeding period would further solidify females as capital breeders and help determine if growth processes are also based on energy stores obtained in prior summers.

Life-history transitions

Although data from Chapter 2 did not suggest that stored energy sources influence the 'decision' to migrate, I do provide evidence that energy stores relate to reproductive behavior in red-sided garter snakes. By using exogenous leptin to mimic larger fat stores, I show that larger fat stores increase reproductive behavior during the spring mating season in both female and male garter snakes. Leading up to spring emergence, the utilization of fat and glycogen stores during hibernation can impact energy stores available for mating activities. Further, an elevated hibernation temperature causes depletion of adipose and glycogen stores to a greater extent than hibernation at 4°C in male snakes (Chapter 4). As elevated energy stores promote mating behavior in garter snakes (Chapter 3), higher hibernation temperatures will likely lower the fitness of male red-sided garter snakes through a decrease in reproductive effort. Conversely, our findings suggest that the fitness of female garter snakes will likely not be affected in the same manner because females do not display the same sensitivity to the depletion of energy stores during hibernation at an elevated temperature as males do. Collectively, this research may explain the variation in reproductive effort exerted by red-sided garter snakes in that individuals that have larger energy stores upon emergence from hibernation have more energy to spend on reproductive activities. This research provides a physiological mechanism that likely contributes to how animals pinpoint the appropriate time to engage in reproductive behaviors and activities from an energy-status perspective, an idea that can be extrapolated to organisms performing intermittent bouts of feeding.

The majority of research examining how energy metrics influence reproduction, including findings from Chapter 3, focuses on behavior and investment in species ranging from lizards to hamsters (e.g., Wade and Schneider 1992; Doughty and Shine 1997, 1998; McInroy et al. 2000). The studies that examine how energy metrics influence reproductive physiology have received far less attention and focus on how adipose metrics influence menstruation and sperm production/mobility in humans (Tataranni et al. 1997; Fabbri et al. 1999; Ziomkiewicz et al. 2008; Chavarro et al. 2010; Ehala-Aleksejev and Punab 2015). Using body weight as a proxy for energy status, Ellison (2003) described the positive relationship between body weight and the reproductive hormones estradiol and progesterone across many human populations. However, changes in body weight do not provide information as to which energy stores may be involved in theses processes. Along with the association that both liver glycogen (Long 1987; Becker et al. 2013) and adipose stores (Doughty and Shine 1997, 1998; Smith and Moore 2003) decrease during reproductive periods, more studies are needed to determine how energy stores influence reproductive physiology across multiple species. Incorporating comparative models into such research is necessary to gain a broader perspective to better understand any biological phenomenon.

By expanding studies to examine how hormones respond to energy stores, we will likely obtain a physiological mechanism to connect reproductive behavior and physiology because hormones have the ability to influence behavior. In addition to sex steroid hormones, other prominent reproductive

hormones such as arginine vasotocin/vasopressin (AVT/AVP), gonadotropin-releasing hormone (GnRH), luteinizing hormone, and follicle-stimulating hormone should be investigated because they may reveal a hormonal mechanism to connect energy status to reproductive behavior. Diet and caloric manipulations provide further evidence to suggest that energy stores impact reproductive hormones (Guezennec et al. 1982; Davies et al. 2015; Valle et al. 2015). Fasting significantly decreases plasma testosterone and luteinizing hormone in male rats (Guezennec et al. 1982). Although food availability does not affect baseline levels of testosterone or luteinizing hormone in Abert's towhees (*Melozone aberti*), responses to GnRH and luteinizing hormone challenges in males on a restricted diet cause smaller increases in plasma testosterone compared to *ad libitum* individuals (Davies et al. 2015). Male house finches (*Haemorhous mexicanus*) with free access to food displayed an initial, significant increase in testosterone (Valle et al. 2015). Interestingly, plasma testosterone levels of *ad libitum* fed individuals returned to baseline after seven weeks of dietary treatment and these levels were statistically nonsignificant from food-restricted individuals. That same study also found that restricting food intake in house finches increased the number of GnRH-immunoreactive neurons. The authors suggest that by decreasing the release of GnRH, the number of GnRH-labeled neurons increased. All this evidence points to a relationship between energy status and the hypothalamus-pituitary-gonad axis. While these studies measured furcular fat, which decreased in response to food restriction (Davies et al. 2015; Valle et al. 2015), neither measured liver glycogen. Without investigations into how food

availability influences multiple forms of stored energy, we cannot understand the preferential usage or loss of one stored energy form over another. Further, direct manipulations of glycogen and fat stores would provide causal evidence as to how energy stores affect reproductive processes, whether they be behavioral or physiological.

It is also possible that different energy stores differentially influence separate aspects of the reproductive axis. For instance, it is possible that liver glycogen stores affect reproductive hormones and fat affects the growth and development of reproductive organs. Although little research to support such a dichotomy has been conducted, male red-sided garter snakes display a significant correlation between testosterone and plasma glucose levels, but not plasma lipids (Crews et al. 1987). Red-sided garter snakes represent a good model to investigate the preferential usage of energy substrates on reproductive processes for a few reasons. First, we can relate the utilization of energy stores over hibernation to sex steroid hormones in both male and female snakes. Significant decreases in adipose stores in males hibernated at 12°C presented in Chapter 4 correlate with decreasing plasma androgen levels during hibernation from Lutterschmidt and Mason (2009), although males from that study were subjected to a 10°C hibernation. Further aligning with our data from Chapter 4, males hibernated at 4°C and females regardless of hibernation temperature did not display significant increases in androgens and estradiol, respectively, from the pre-hibernatory period up until 16 weeks in hibernation (Lutterschmidt and Mason 2009).

Future directions

Expanding energetic studies to include measurements of organ size would provide a more comprehensive view of an organism's energy status and insight into potential trade-offs between hyperplasia (i.e., increase in cell number) and hypertrophy (i.e., increase in cell size), particularly in adipose tissue. Until recently, evidence suggested that the number of adipocytes was primarily determined during embryonic development (Spalding et al. 2008; Hirsch and Han 1969). With the ability to increase the number of fat cells in adults (Wang et al. 2013; Vishvanath et al. 2016), there is likely a balance between hypertrophy and hyperplasia in that adipocytes may increase their size to accommodate excess calories until enough energy is stored to invest in cell proliferation. However, increasing adipocyte size can lead to hypoxic conditions that can induce apoptosis (Bozec and Hannemann 2016) and negatively impact metabolic health such as increasing susceptibility to diabetes (Ghaben and Scherer 2019). Because red-sided garter snakes routinely change adipocyte size on an annual basis, investigating the potential trade-offs between hyperplasia and hypertrophy of adipose cells in these snakes could provide insight into treatments for detrimental metabolic conditions.

The red-sided garter snake system also represents a unique opportunity to investigate how energy stores differentially influence reproductive processes. Northern populations of red-sided garter snakes are dissociated breeders where peak mating behavior does not coincide with peak levels of sex steroid hormones or the maturation of gametes (Crews 1984; Crews et al. 1984; Krohmer et al.

1987; Whittier et al. 1987; Moore et al. 2000; Moore and Mason 2001; Lutterschmidt and Mason 2009). Because of this dissociation, we can ask whether adipocyte follicle size or liver glycogen differentially influences sex steroid hormones, reproductive behavior, and the maturation of gametes separately. Because its homolog mediates reproductive behavior in mammals, arginine vasotocin (AVT) is one neuropeptide that may provide a hormonal mechanism to connect energy stores to behavior in red-sided garter snakes. Further, there are seasonal differences in the number of AVT-immunoreactive neurons with males and females displaying more hypothalamic AVT-labeled cells in the spring compared to the fall (Lucas et al. 2017). With the variation in the levels of brain AVT, we can determine if either adipocyte follicle size or liver glycogen explains the variation in AVT-immunoreactive neuron number. Examining if one stored energy form over another influences specific reproductive processes could provide insight into the treatment of reproductive issues such as infertility in humans.

Despite the association of energy status with reproduction across taxa and specifically in red-side garter snakes, I did not find evidence to suggest that adipocyte follicle size or liver glycogen influence the transition from mating to feeding behavior in garter snakes. This eliminates yet another possible physiological mechanism thought to be involved in signaling the switch from mating to feeding behavior in red-sided garter snakes, a focal area of research in the Lutterschmidt lab (Cease et al. 2007; Lutterschmidt and Maine 2014; Lucas 2015; Dayger and Lutterschmidt 2017). It is possible differences in reservoir

energy substrates with migratory status were not identified because tissue-specific energy stores may be more important in signaling the appropriate time to migrate. The energetic demands of skeletal muscles during the spring mating season are high, particularly in male red-sided garter snakes. Male snakes spend up to two to three weeks courting and attempting to mate with females at the den (Shine et al. 2001; Lutterschmidt and Mason 2009). In addition to locomoting to search for females, males routinely employ the courtship behaviors of caudocephalic waving and tail searching to attempt to copulate with females. Further, male metabolic rate significantly increases with increasing courtship intensity (Friesen et al. 2017). Competition to mate with females is fierce, with mating balls consisting of upwards of 100 males and one female (Crews and Garstka 1982), further adding to the energetic demands of skeletal muscle. Even after leaving the den, snakes must utilize skeletal muscle to migrate upwards of 17 km to summer feeding grounds for replenishing energy stores (Gregory and Stewart 1975; Gregory 1977). With such an energetically demanding usage of skeletal muscles at the end of a long aphagic period, it is possible that the depletion of energy stores in skeletal muscles may influence the decision to migrate in male red-sided garter snakes, an idea that has yet to be tested. A few studies support the idea that tissue-specific energy stores may influence behavioral switching because stored fat in skeletal muscle decreases during migration in Chinook salmon (Mesa and Magie 2006; Bowerman et al. 2017). Investigations of energy stores at the tissue level should be conducted to

determine if fat and glycogen stores are involved in transitions between mating and feeding behaviors in red-sided garter snakes.

As with reservoir adipose stores and, to a lesser degree, liver glycogen, sex-specific differences likely occur in the stored energy of tissues in red-sided garter snakes. First, male red-sided garter snakes have significantly higher metabolic rates than females following emergence from simulated hibernation (Crews et al. 1987). Females likely have a lower energy demand in the usage of skeletal muscles during spring mating as compared to male red-sided garter snakes. While males spend up to two weeks at the den, females migrate away from the den within 24 hours of emergence (Lutterschmidt and Mason, 2009; Shine et al., 2001; Wilson and Lutterschmidt, unpublished data). Although females do not perform caudocephalic waves, they expend energy to evade males' attempts at copulation as they move away from the den after emergence. As females migrate away from the den, their movements are impeded by numerous males courting and attempting to mate with them (Shine et al. 2004), thereby increasing the energy demand on a female's skeletal muscles. However, neither males nor females elevate plasma lactic acid levels in response to courting, only mated snakes significantly increase plasma lactic acid levels compared to snakes not engaging in mating behaviors (Shine et al. 2004). These sex-specific differences in the utilization of skeletal muscles likely translate to different energy demands and therefore energy stores at the tissue level.

Findings from Chapters 2 and 4 bolster the hypothesis that stored energy in skeletal muscle influences the decision to migrate in red-sided garter snakes.

First, migrating females have a significantly lower body condition index than non-migrating females. Although only investigated in male red-sided garter snakes, skeletal muscle is important in determining body condition as lean mass (e.g., muscle, skeleton) contributes significantly to body condition, while fat and liver masses do not (Shine and Mason 2005). If skeletal muscle also significantly contributes to body condition in female garter snakes, it's likely that the depletion of energy stores in skeletal muscles influences the decision to migrate in both female and male red-sided garter snakes. Additionally, prior to spring emergence, body condition index in female snakes significantly decreases over hibernation, whereas male body condition nonsignificantly decreased. A significant decline in body condition index at the time of spring emergence in female but not male red-sided garter snakes may explain, in part, why females migrate away from the den much sooner than males. And as females migrate further away from the den, they likely continue to deplete energy substrates in their skeletal muscle as evidenced by a lower body condition index at the road compared to the den. All of this evidence points to the necessity of investigating muscle tissue to determine its involvement in the decision to migrate in red-sided garter snakes.

Until research investigating energy stores in muscle tissues is conducted, we won't know if garter snakes adhere to the idea of fueling muscles from internal energy stores for migratory activities. However, our findings, along with other reptilian research, suggest taxonomic differences between reptiles and mammals in the energy substrate primarily utilized to fuel physiological

processes during hibernation. In addition to the discussion of differences in thermal ecology between ectotherms and endotherms in Chapter 4 (pages 116-117), perhaps other physiological limitations in endotherms influence the different usage of energy substrates to meet energy demands during hibernation. While mammals keep plasma glucose levels within a narrow range to maintain homeostasis, reptiles allow significant fluctuations in plasma glucose levels (Secor and Carey 2016). Perhaps allowing greater fluctuations of plasma glucose levels in reptiles relates to more flexibility in limiting the usage of glucose to ensure the continued function of tissues that exclusively utilize glucose to fuel cellular processes such as the central nervous system, kidney and blood cells (Reinmuth et al. 1965). Despite the established theory that organisms employ glucose sparing techniques to ensure essential organs are fueled during times of fasting or starvation, investigations into which energy substrates are utilized in nonmammals are lacking. Investigations into the usage of energy substrates in a tissue-specific manner outside of mammals will allow the determination of adherences to or divergences from established paradigms presented in traditional models. Further, such investigations could expand our knowledge of the physiological capabilities of organisms and may provide foundational research into how these processes can be commandeered for human health benefits.

Gaining a wider perspective into the spectrum of physiological phenomenon across taxa ultimately informs the vast capabilities of all bodies including our own. My dissertation aimed to add to this spectrum by examining

how stored forms of energy differ across varying life-history stages in an organism with the ability to endure prolonged and predictable bouts of fasting. I've uncovered similarities and differences in the usage of energy substrates to fuel physiological processes as compared to other species, but particularly mammals. As the majority of research on energy substrates is correlative rather than causative, much research still remains to be conducted to further determine how energy substrates influence an organism's physiology and behavior. Pursuing such research will not only broaden our knowledge of the diverse ways in which an organism is able to survive, but may also provide insights into human health from an energy standpoint. Lastly, further investigations into how energy status fluctuates with the environment will provide insight into organismal fitness that certainly has implications for management and conservation efforts.

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