AN ABSTRACT OF THE DISSERTATION OF

Emily J. Uhrig for the degree of Doctor of Philosophy in Zoology presented on April 21, 2015.

Title: Reproductive Implications of Parasitic Infections and Immune Challenges in Garter Snakes

Abstract approved: ______________________________________________________

Robert T. Mason

Parasitic infections and immune challenges can affect host reproductive fitness and, ultimately, the evolution of host populations in a myriad of ways. The fitness implications of parasitic infections range from increased host mortality to subtle changes in reproductive investment. From alterations of behaviors, sexual signaling, and competitive ability to changes in gamete production and fertilization success, it is clear that parasites are capable of mediating sexual selection and influencing host reproductive fitness even without altering mortality. The mechanisms underlying fitness effects highlight the complexity of the host-parasite relationship which involves immune responses as well as a range of other, often interactive, physiological processes within the host. In some instances, it is not the direct effect of parasites per se, but rather the hosts’ responses to infection that mediate fitness consequences. This dissertation presents studies designed to elucidate the implications of parasitism and immune responses for the reproductive fitness of garter snakes (genus *Thamnophis*).
In chapter 2, “Alaria mesocercariae in the tails of red-sided garter snakes: evidence for parasite-mediated caudectomy”, I focus on the histopathological changes associated with a trematode (Alaria sp.) infecting the tails of red-sided garter snakes (T. sirtalis parietalis). My results demonstrate that Alaria mesocercariae occur in high density within the tail tissue of both male and female snakes with as many as 2,000 mesocercariae in a single tail; infection prevalence was 100% in the snakes I examined. I found no evidence of intersexual variation in pathological changes or infection densities. For both sexes, external pathological manifestations include swelling of the tail while, internally, the aggregation of mesocercariae leads to the formation of mucus-filled pseudocysts and damage of muscle tissue. In severe cases, the extent of tissue destruction appeared to weaken the connection of the tail to the rest of the body, a condition that would facilitate tail breakage, which in turn negatively affects the snake’s fitness by impairing mating success. From the parasite’s perspective, tail breakage is likely beneficial by facilitating its transmission to subsequent hosts in its life cycle.

Alaria sp. are not the only parasites commonly infecting garter snakes and in chapter 3, “Patterns in parasitism: interspecific and interpopulational variation in helminth assemblages and their reproductive fitness correlates in garter snakes”, I broaden our investigation to include a suite of helminth parasites common in the garter snakes of Manitoba, Canada. My results demonstrate that helminth assemblages of two garter snake species (red-sided garter snakes, T. sirtalis parietalis, and plains garter snakes, T. radix) include Lechriorchis trematodes and Rhabdias nematodes in the lung, Alaria mesocercariae in the tail, and diplostomid trematode metacercariae in the visceral fat; red-sided garter snakes also had gastrointestinal cestodes. Helminth assemblages
varied, mainly in terms of parasite density, among populations of red-sided garter snakes and between red-sided and plains garter snakes, but it is unclear whether this variation is due simply to diet-based differences in parasite exposure or whether variation in parasite resistance may have a role. Notably, for plains garter snakes and one red-sided garter snake population I found helminth densities to be predictive of male fitness correlates, namely body condition, testes mass, and sperm counts. Thus, parasitism in garter snakes clearly has important implications for reproductive fitness beyond just influencing tail loss. These results highlight the importance of considering more than a single parasite or single fitness correlate when exploring host-parasite relationships.

The consequences of parasitic infections may arise simply through the activation of the host’s immune system rather than the presence of parasites. Thus, in chapter 4, “Changes in reproductive investment and hormone levels in response to an acute immune challenge”, I use lipopolysaccharide (LPS) to assess immune-reproductive tradeoffs of male red-sided garter snakes during the breeding season. As LPS is non-pathogenic, I was able to assess the fitness implications of the immune activation itself. My results showed that males depress courtship behaviors and mating success when faced with a single acute immune challenge. For LPS-treated males that did mate, copulatory plug mass was significantly lower compared to controls, while sperm counts did not differ between treatments. This result likely reflects the dissociated breeding pattern of these snakes as spermatogenesis occurs outside the breeding season and, thus, sperm stores were already in place prior to the immune challenge whereas plug material is produced during the breeding season. Further, the LPS treatment was correlated with increased plasma levels of corticosterone, which were 1.8 times higher in LPS-treated males
compared to controls, and decreased levels of androgens, which, in LPS-treated males, were only one third as high as androgen levels in control males. Thus, the observed immune-reproduction tradeoff appeared to be hormonally-mediated. Indeed, the low breeding season androgen levels characteristic of this dissociated breeder may have relaxed testosterone-mediated immunosuppression and so facilitate immune-induced suppression of reproductive behaviors. The results of this study highlight the influence of host life history on the consequences of immune activation and also emphasize the complex interactions between the immune, reproductive and endocrine systems.

In chapter 5, “Implications of repeated immune challenges in a capital breeder with prolonged hibernation”, I again utilized LPS as a means of investigating the implications of immune activation. In this study, I administered a series of LPS injections to male and mated female snakes throughout the summer feeding season, and, for males, into the autumn. Females give birth during the summer and males undergo testicular recrudescence and spermatogenesis during summer and into autumn so these seasons represent important reproductive periods for red-sided garter snakes. Also, as capital breeders, it is during the summer feeding season that snakes of both sexes accumulate the resources upon which they will rely throughout hibernation and the subsequent breeding season. For the most part, my results did not demonstrate clear immune-reproductive tradeoffs. It appears that the absence of tradeoffs may be due to immune-challenged males and gravid female compensating for the immune challenge and maintaining reproductive processes by increasing their food intake, which was not limited during the study. Indeed, LPS-treated gravid females actually had more offspring per litter compared to gravid control females, suggesting that the immune challenge led to greater
investment in offspring. In contrast to gravid females, non-gravid females treated with LPS exhibited reduced food intake which may reflect a survival strategy as anorexia during infections tends to be beneficial for survival. Interestingly, the increased food consumption of males did not translate into greater fat stores, but rather higher liver masses which may be indicative of immunopathological changes which should be explored in future studies.
Doctor of Philosophy dissertation of Emily J. Uhrig presented on April 21, 2015

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

_____________________________________________________________________

Emily J. Uhrig, Author
ACKNOWLEDGEMENTS

To my mom and dad, and Uncle Donald for enduring support and encouragement of a waterdog girl.
CONTRIBUTION OF AUTHORS

For Chapter 2: E. J. Uhrig developed the initial study design, collected and preserved all tissue samples, conducted non-histological lab work, conducted all statistical analyses, and wrote the majority of the manuscript. S. T. Spagnoli prepared histological slides and histology images, contributed to the corresponding methods section and provided histopathological descriptions for the results. M. L. Kent assisted with study design and R. T. Mason assisted with animal collection.

For Chapters 3, 4, and 5: E. J. Uhrig designed the studies, performed dissections, collected data and processed sperm samples, conducted all statistical analyses and wrote the manuscript. C. R. Friesen and L. A. Blakemore assisted with data collection. R. T. Mason assisted with animal collection. For chapter 4, D. I. Lutterschmidt collected blood samples and conducted radioimmunoassays.
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Chapter 1: General Introduction

Host-parasite relationships and the associated immune responses are important drivers of evolution for both host and parasite species via natural and sexual selection (Hamilton and Zuk 1982; Minchella 1985; Howard and Minchella 1990; Lafferty 1993; Folstad and Karter 1992; Able 1996; Poulin and Vickery 1996; Penn and Potts 1998; Boots and Haraguchi 1999; Poulin and Thomas 1999; Duffy and Sivars-Becker 2007). Thus, it is important that the effects of parasitic infections be considered when seeking to understand evolutionary processes of a population. Yet our understanding of the effects of parasitism on host fitness is relatively limited for many wild species compared to domesticated and livestock species. Additionally, there is clearly a taxonomic bias among studies of parasitic infections in vertebrates (e.g. see recent meta-analyses: Poulin and Forbes 2012; Watson 2013). Compared to the body of literature on avian and mammalian host-parasite relationships, there are relatively few studies of parasitism in reptiles and this paucity of studies is particularly apparent with regard to snake species. This taxon bias generally extends to immunological studies as well despite the burgeoning field of ecoimmunology highlighting the role of variation in immune responses with regard to evolutionary processes. It is clear, therefore, that investigations focusing on the implications of parasitic infections and immune responses in wild taxa, and particularly reptilian species, are crucial if we are to understand the broader patterns of life histories and evolution in these organisms.
1.1 Parasites Defined

There is some ambiguity around the term ‘parasite’ in the biological literature with individual authors often delineating the definition in their own ways to include or exclude certain groups of organisms (Crofton 1971; Zelmer 1998; Bush et al. 2001; Combes 2001; Poulin 2006). However, the most generally recognized definition describes parasitism as a symbiotic relationship (i.e. a close physical association) between organisms where one, the host, is harmed in some way due to the presence of the parasite which in turn benefits from the interaction (Bush et al. 2001; Poulin 2006). The ‘harm’ to the host occurs in the form of reduced fitness in which the ability of the host to pass his or her genes on to the next generation is compromised to some degree. The term parasite is sometimes reserved for eukaryotic organisms to the exclusion of bacteria and viruses (e.g. Zimmer 2000; Bush et al. 2001). However, many biologists include pathogenic bacteria, fungi, and viruses among parasites as so called microparasites (Anderson and May 1979; Bush et al. 2001; Combes 2001). In addition to bacteria and viruses, microparasites also include the smallest of the more traditional parasites such as intracellular protozoa (Anderson and May 1979; Bush et al. 2001). Macroparasites, then, are the larger scale parasites such as helminths and arthropods (Anderson and May 1979; Bush et al. 2001). A further distinction that is often made is that between parasite and pathogen where pathogen is defined by the ability to cause damage or disease (Falkow 1997; Casadevall and Pirofski 1999; Brown et al. 2006; Méthot and Alizon 2014). Thus, not all parasites are considered pathogens, but often all pathogens are considered parasites (Brooks and Hoberg 2006; Johnson and Hoverman 2012; but see Bos and Parlevliet 1995; Kosoy 2013). Here it
is worth noting that while the aforementioned definitions have their utility, they should not necessarily be considered discreet categories. Rather the nature of the symbiotic relationship (e.g. mutualistic vs. parasitic) or pathogenicity (e.g. pathogenic vs. non-pathogenic) are arguably better thought of as continuums (Sapp 2004; Kosoy 2013; Méthot and Alizon 2014). Further, whether a particular organism is considered parasitic and/or pathogenic can depend greatly on the ecological context in which it is found (Casadevall and Pirofski 1999; Sapp 2004; Kosoy 2013; Méthot and Alizon 2014).

1.2 Parasitism and Host Reproduction:

Host mortality is clearly a severe outcome for a parasitic infection and one that can be detrimental to the parasite’s fitness as well as the host’s if, for example, host death curtails parasite transmission (Anderson and May 1982; Antia et al. 1994). Thus, parasites may evolve toward an optimal virulence such that they exploit host resources at a rate which is beneficial to parasite fitness while not killing the host too quickly so that transmission can be maximized (Anderson and May 1982; Antia et al. 1994; Ebert and Herre 1996; Jensen et al. 2006). Survival is essential to host fitness; clearly, at least if any degree of reproduction is possible, even an individual with a heavy parasite burden has greater fitness than a dead individual. However, the often subtle sub-lethal effects of parasitic infections can still result in fitness reductions for the host. Parasites, for example, can mediate competitive and predator-prey interactions between hosts (Price et al. 1986; Schall 1992; Hatcher et al. 2006; Kelly et al. 2009). Schall (1992), for instance, found that malarial parasites mediate
competitive interactions between two species of *Anolis* lizards. *A. gingivinus* is more often infected by malaria compared to *A. wattsi* and, in locations where malaria infections occur, both *Anolis* species exist, but in malaria-free locations, only *A. gingivinus* is present (Schall 1992). Parasitic infections, however, do not only mediate interspecific interactions. As discussed in the following paragraphs, parasites can also affect host reproduction either by direct effects of the parasite or via activation of the host immune system.

Perhaps the most extreme examples of parasites affecting host reproduction are the parasitic castrators where host gonads are either destroyed or effectively shut down by the presence of a parasite (Baudoin 1975; Lafferty and Kuris 2009). In cases where parasitism leads to host castration before successful reproduction occurs, the host contributes nothing to the gene pool and is functionally dead from an evolutionary perspective (Baudoin 1975; Lafferty and Kuris 2009). In response to the selective pressure of a castrating parasite, some snails (e.g. populations of the California horn snail, *Cerithidea californica*) have evolved strategies such as earlier sexual maturation to minimize the infection’s consequences (Lafferty 1993). Thus, in some instances, the presence of a parasite can alter major life history events of the host.

In somewhat less extreme examples, parasites can act as mediators of sexual selection as highly parasitized individuals may, for instance, be less sexually attractive or inferior in competitive mating interactions (Hamilton and Zuk 1982; Clayton 1991; Zuk 1992; Andersson 1994; Møller, Christe, and Lux 1999; Grammer et al. 2003). Classic examples of parasite mediated sexual selection include the
negative correlations between bright coloration and parasite burden observed in many avian and fish taxa (e.g. Hamilton and Zuk 1982; Milinski and Bakker 1990; McGraw and Hill 2000; Mougeot et al. 2007; Maan et al. 2008). Parasite-mediated sexual selection is not limited to the visual modality, however. Rather, variation in acoustic signals and chemical signals based on parasite burden occur as well (Penn and Potts 1998; Buchanan et al. 1999; Maksimowich and Mathis 2001; Madelaire et al. 2013). For example, Madelaire et al. (2013) found that parasite burden was negatively correlated with calling rate for male tree frogs (*Hypsiboas prasinus*). The benefit of recognizing a potential mate’s parasite burden prior to copulation may include both genetic benefits (e.g. heritable parasite resistance for offspring) as well as more direct benefits such as avoiding acquisition of parasites (Hamilton and Zuk 1982; Able 1996; Roulin et al. 2001; Kavaliers et al. 2004).

In addition to influences on pre-copulatory sexual selection, parasites can alter reproductive fitness in the post-copulatory setting. Sperm of infected males, for example, may be at a competitive disadvantage in the reproductive tract of females that have mated with multiple males during the breeding season (Poiani 2002; Champion de Crespigny and Wedell 2006; Kekäläinen et al. 2014; Kaufmann et al. 2014). Additionally, infected individuals that do successfully produce offspring may nonetheless produce fewer or lower quality offspring (Worden et al. 2000; Sanz et al. 2001; Knowles et al. 2010). For female pied flycatchers (*Ficedula hypoleuca*), for example, infection by blood parasites (*Haemoproteus balmorali*) led to reduced egg hatching success (Sanz et al. 2001).
Although by definition parasites depress host fitness, not all parasitic infections lead to inhibition of reproduction. Indeed, infected hosts may in some circumstances exhibit increased reproductive efforts such as increased sexual activity (Forbes 1993; Polak and Starmer 1998; Agnew et al. 2000). Such instances may occur when the parasitic species can raise its own fitness by increasing contact among hosts as is the case for sexually transmitted parasites (Lombardo 1998; Klein 2003; Knell and Webberley 2004). Alternatively, increased reproductive efforts can represent a terminal investment type strategy where the host attempts to maximize reproduction before the infection progresses to castration or death (Thornhill et al. 1986; McCurdy et al. 2000). Regardless of the direction of the effect, it is clear that parasitism has the potential to influence the evolutionary trajectory of a species via alterations of host reproductive fitness. At the more proximate level, it should be recognized that the effects of parasites may arise not only via the direct effects of the parasites, but also via the immune responses of the host to the parasites.

1.3 Parasitism and the Immune System:

Studies of parasitism and host reproductive fitness would be incomplete without acknowledging the role of the immune system. Indeed, Møller et al. (1999) suggested that examining immune function could be a “powerful” approach to assessing parasite mediated sexual selection. Also, if a particular parasite and some measure of host reproductive fitness are found to be correlated, it may not indicate a direct result of the parasite per se, but instead may actually reflect the consequences of the host’s immune response (Sheldon and Verhulst 1996; Schmid-Hempel 2003).
For example, experiments exposing hosts to isolated antigens, thus eliminating direct parasite effects, have demonstrated that an immune response alone is capable of reducing host reproductive fitness (e.g. fewer eggs produced – Ahmed et al. 2002; decreased parental care, fewer fledglings – Ilmonen et al. 2000; Bonneaud et al. 2003) and such immune-reproduction tradeoffs have been noted in a variety of taxa. Adding another layer of complexity, the immune system itself is influenced by additional factors such as hormone levels which in turn may affect or be affected by parasitism (Lawrence 1986; Dunlap and Schall 1995; Roberts et al. 2001; Escobedo et al. 2005). Thus, there is a complex interplay between parasitism, physiology, and host reproduction that must be considered if one is to understand the fitness consequences of parasitic infections.

1.4 Parasites of Reptiles:

In addition to facilitating a better understanding of reptilian evolutionary and behavioral ecology, studies of host-parasite relationships and the associated immunology in reptilian species are likely to have even broader significance by shedding light on the understanding of the evolution of the vertebrate immune system (Zimmerman et al. 2010). Indeed, in their 2010 review, Zimmerman et al. describe reptiles as a “pivotal group to study” for elucidating vertebrate immune system evolution. However, despite their relevance, studies of host-parasite relationships in reptiles are few in comparison to mammalian, avian, or invertebrate taxa. Of the reptilian studies that have been conducted, the majority of work has focused on lizards (e.g. *Lacerta* spp. – Sorci et al. 1996; Václav et al. 2007; *Podarcis* spp. –
Huyghe et al. 2010; Carretero et al. 2011; Anolis spp. - Schall and Staats 1997; Schall et al. 2000) and often on malarial parasites (Schall et al. 1982; Dunlap and Schall 1995; Schall et al. 2000; Scholnick et al. 2010). In comparison to lizards, parasitism and immunology in snakes is even less often studied and this paucity extends even to otherwise very well studied taxa such as the garter snake genus *Thamnophis*. The dearth of studies of snake parasites becomes particularly concerning in light of emerging infectious diseases such as the snake fungal disease (SFD) caused by *Ophidiomyces ophiodiicola* (formerly *Chrysosporium*) that infects wild snakes, including *Thamnophis* spp. (Dolinski et al. 2014), in the midwestern and eastern United States (Nichols et al. 1999; Allender et al. 2011; Latney and Wellehan 2013; Mitchell and Walden 2013; Sleeman 2013).

1.5 Garter snakes as models for studying fitness consequences of parasitic infections and immune challenges

Parasites of garter snakes have been mentioned in the literature as early as 1914 when Evermann and Clark noted the presence of nematodes in the stomach of *Thamnophis sirtalis*. However, in the course of the ensuing century, studies of parasites in any garter snake species have been limited mainly to parasitological surveys with little or no discussion of possible fitness consequences for the host (e.g. Gibson and Rabalais 1973; Rau and Gordon 1978; Goldberg and Bursey 2002; Jiménez-Ruiz et al. 2002). Immunological studies of garter snakes are similarly sparse with only a handful of such studies, mostly of the western terrestrial garter snake (*T. elegans*), apparent in the literature (e.g. Coe et al., 1976; Sparkman and
Palacios, 2009; Palacios et al., 2011). With regard to red-sided garter snakes (*T. sirtalis parietalis*), the primary focus of this dissertation, parasites have previously been noted in this subspecies (e.g. Wacha and Christiansen, 1974; Gregory, 1977); however, no studies have specifically focused on the fitness implications of parasitic infections or immune responses in red-sided garter snakes despite this subspecies of *Thamnophis sirtalis* being the focus of numerous behavioral and physiological studies.

The reproductive biology of the red-sided garter snake has been particularly well-studied over the past 40 years. Studies have focused on its reproductive ecology and mating system (Gregory 1974, 1977; Gregory and Stewart 1975; Shine et al. 2001), sexual selection and sexual conflict (Shine et al. 2000; Shine et al. 2004a; Friesen et al. 2013; Friesen et al. 2014a,b), courtship and copulatory behaviors (Pisani 1976; Crews et al. 1984; O’Donnell et al. 2004; Shine et al. 2004b; Shine et al. 2012), neurobiology (Friedman and Crews 1985; Krohmer and Crews 1987; Mendonça et al. 2003; Krohmer et al. 2011; Lutterschmidt and Maine 2014), endocrinology (Crews 1976; Whittier et al. 1987; Moore et al. 2000; Moore and Mason 2001; Lutterschmidt et al. 2004; Lutterschmidt and Mason 2005, 2009), and chemical ecology (Mason et al. 1989, 1990; LeMaster and Mason 2001, 2002; Parker and Mason 2009; including several of my own publications not included within this dissertation: Uhrig et al. 2012, 2013, 2014). However, despite the considerable body of knowledge concerning garter snake reproduction, no studies of garter snakes have focused on the reproductive implications of parasitic infections, and immune-reproductive tradeoffs have received very little attention. Given the extensive understanding of garter snake
reproduction, these animals have great potential for broadening our understating of how parasitic infections can influence host reproductive fitness and, ultimately, host evolution. Thus, by investigating parasitism and reproductive-immune tradeoffs in garter snakes, my dissertation aims to elucidate an underexplored and poorly understood area of reptile biology.

1.6 References:


Hormonal alterations and reproductive inhibition in male fence lizards (Sceloporus occidentalis) infected with the malarial parasite Plasmodium mexicanum. Physiological Zoology, 608–21.


Chapter 2: *Alaria mesocercariae* in the tails of red-sided garter snakes: evidence for parasite-mediated caudectomy

Emily J. Uhrig, Sean T. Spagnoli, Michael L. Kent, and Robert T. Mason

Abstract:

Trematodes of the genus *Alaria* develop into an arrested stage, known as mesocercariae, within their amphibian second intermediate host. The mesocercariae are generally transmitted to a non-obligate paratenic host before reaching a definitive host where further development and reproduction can occur. Snakes are common paratenic hosts for *Alaria* spp. with the mesocercariae often aggregating in the host’s tail. The current study aims to assess the prevalence, intensity, and pathological changes associated with *Alaria* spp. mesocercariae in the tails of red-sided garter snakes (*Thamnophis sirtalis parietalis*). Infection prevalence was 100% for both male and female snakes. Infection intensity ranged from 11 to more than 2,000 mesocercariae per snake tail, but did not differ between the sexes. External pathological changes included tail swelling while histopathological changes included mild inflammation and the presence of mucus-filled pseudocysts surrounding mesocercariae, as well as the compression and degeneration of muscle fibers. Our results indicate that mesocercariae can lead to extensive muscle damage and loss in both sexes which likely increases the fragility of the tail making it more prone to breakage. As tail loss in garter snakes can affect both survival and reproduction, infection by *Alaria* mesocercariae clearly has serious fitness implications for these snakes.
2.1 Introduction

*Alaria* is a genus of digenetic trematodes with a life cycle that is unusual among trematodes in that it includes a mesocercarial larval stage that generally passes through one or more paratenic hosts (Bosma 1934; Möhl et al. 2009). As with most trematodes, eggs from adult *Alaria* spp. passed from the digestive tract of the definitive host hatch into miracidia which infect aquatic snails as first intermediate hosts (Bosma 1934; Pearson 1956; Möhl et al. 2009). Cercariae emerging from a snail next enter a frog where, rather than developing into encysted metacercariae as is typical of trematodes in their second intermediate hosts, they develop into mesocercariae (Bosma 1934; Pearson 1956; Möhl et al. 2009). This mesocercarial stage, first described by Bosma (1934), is a non-reproductive stage that will not undergo further development into the metacercarial and adult stages until ingested by a suitable definitive host, a mammalian carnivore such as a mustelid or canid (Möhl et al. 2009). Frequently, however, the infected frog is consumed by a paratenic host in which the mesocercariae will persist unchanged (Shoop 1988; Möhl et al. 2009). As a paratenic host ingests more mesocercariae, the larvae accumulate in the host’s tissues increasing the number of parasites that can be transmitted to the definitive host. Thus, while the paratenic host is not physiologically necessary for the completion of the *Alaria* life cycle, it can have an ecologically important role in parasite transmission (Möhl et al. 2009).

The mesocercarial stage of *Alaria* spp. has low host specificity and a variety of paratenic hosts have been reported including snakes, opossums, raccoons, and rodents as well as game species such as wild boar and alligator (Shoop and Corkum
1981; Möhl et al. 2009). Via consumption of mesocercarial infected meat, humans can also become paratenic hosts (Freeman et al. 1976; Möhl et al. 2009). Indeed, alariosis has been cited as a (re-)emerging zoonosis of particular concern in Europe (Tăbăran et al. 2013; Wasiluk 2013; Portier et al. 2014). Within humans, the pathological effects of *Alaria* spp. mesocercariae can be serious and at least one fatal infection involving severe pulmonary hemorrhaging has been documented (Fernandes et al. 1976; Freeman et al. 1976). Other than human case studies, relatively few investigations have specifically addressed the pathological changes associated with infection by *Alaria* spp. in paratenic hosts. This paucity of studies is particularly evident for non-mammalian species such as snakes although they appear to be some of the most commonly documented paratenic hosts.

The current study focuses on *Alaria* spp. infections in red-sided garter snakes (*Thamnophis sirtalis parietalis*) from Manitoba, Canada. Infection by *Alaria* spp. have been frequently noted for a number of *Thamnophis* species including *T. sirtalis* (Rau and Gordon 1978; Goldberg and Bursey 2002; Jiménez-Ruiz et al. 2009). Several studies indicate the propensity for mesocercariae to accumulate in the tails of snakes and observations of gross external changes consisting of a swollen tail with potential tissue necrosis have been noted (La Rue 1917; Olivier and Odlaug 1938; Odlaug 1940; Sparkman and Palacios 2009). In the field, garter snakes are often observed to have lost portions of their tails and it is believed that some such injuries may be facilitated by parasite infection (La Rue 1917; Shine et al. 1999). However, no prior studies have provided detailed investigations of *Alaria* pathological changes in the tails of garter snakes despite the importance of the tail in garter snake survival.
and reproduction. In their study of tail loss patterns in *Thamnophis*, Willis et al. (1982) suggest that juveniles suffering tail loss were less likely to survive the stress of hibernation. With regard to reproduction, males utilize their tails in intrasexual tail wrestling competitions as they compete for access to the female’s cloaca. Shine et al. (1999) found that males missing portions of their tails have reduced mating success. For female snakes, tail loss may make it more difficult for males to determine the location of the female cloaca (Pisani 1976; Perry-Richardson et al. 1990). Thus, parasite mediated tail loss could substantially affect garter snake fitness particularly since, unlike many lizard species, these snakes are not capable of tail regeneration (Fitch 2003).

In the current study, we examined the tails of red-sided garter snakes naturally infected with *Alaria* spp. As the mesocercarial stage is morphologically nearly indistinguishable among species of *Alaria*, species level identification is not possible based on microscopy alone. Preliminary molecular analyses suggest the snakes to be coinfected with *A. mustelae* and *A. marcianae* (V. Tkach, pers. comm.), but for the purposes of the current study we will continue to refer to the parasite as ‘*Alaria* spp.’. The goals of the present study were to 1) characterize the histopathology of *Alaria* infection in garter snake tails, 2) determine the prevalence and intensity of the infections, and 3) determine whether there was evidence of intersexual variation in infection patterns or pathological changes.
2.2 Methods

2.2.1. Study Population

All red-sided garter snakes used in this study were wild caught individuals collected from a den site in the Interlake region of Manitoba, Canada (50°31’58”N; 97°29’71”W); the den is estimated to overwinter approximately 35,000 garter snakes (Shine et al. 2006). Collections were made by hand during the boreal spring breeding season when snakes were emerging from winter hibernation. Snakes remain aphagous throughout hibernation and the breeding season (Aleksiuk and Stewart 1971) thus any Alaria spp. mesocercariae contained within them were acquired during feeding in previous summers. Following their use in a separate behavioral study, snakes (N = 16 males, 16 females) were euthanized with an injection of Beuthanasia® and their tails were immediately collected for histology and parasite counts. All procedures utilized in this research were approved by the Oregon State University Animal Care and Use Committee (ACUP no. 4317). This research complied with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was carried out under the authority of Manitoba Wildlife Scientific Permit WB12405.

2.2.2 Histopathology

Tails collected (N = 6 males, 6 females) for histology were severed approximately 1 cm anterior to the cloacal opening and injected ventrally at 1cm intervals along their length with Dietrich’s fixative. The tails were then stored in additional Dietrich’s until histological analyses at Oregon State University. Tails
were sectioned transversely and decalcified using Cal-Ex II solution. Following
decalcification, tails were embedded in paraffin blocks and processed in the standard
manner. Sections were placed on glass slides and stained using the standard H&E
method. Slides were then evaluated via light microscopy.

2.2.3. Parasite Counts

Tails utilized for parasite counts (N = 10 males, 10 females) were a randomly
selected subset of intact tails collected as part of a separate larger study. Tails were
severed 1 cm posterior to the cloacal opening and the ventral surface was carefully
slit open with scissors. Tails were stored individually in 5% formalin for later
counting. Prior to counting, mesocercariae were separated from tail tissue using fine­
tipped forceps under a stereomicroscope (Nikon SMZ-745T). Mesocercariae were
easily recognized by their characteristic oval shape as well as their conspicuous
penetration glands and acetabulum (Figure 2.1). However, as the mesocercarial stage
is morphologically nearly indistinguishable among species of Alaria, we did not
attempt to differentiate Alaria species during parasite counts. After the tail tissue was
removed, total parasite counts were conducted using a Bogorov-type plankton
counting chamber under the stereomicroscope. Prevalence (percentage of individuals
infected) and intensity of infection (number of parasites per infected host) were
determined (Bush et al. 1997). In order to account for variation in tail length, intensity
was also calculated as the number of mesocercariae per cm of tail for infected
individuals.
2.2.4. Statistics

As the non-normality of tail length data could not be corrected by statistical transformations, tail lengths were compared between sexes using Mann-Whitney Rank Sum tests. Male and female infection intensities were compared using Student’s $t$-tests. All statistical tests were conducted in the SigmaPlot software package (version 12.5; Systat Software, Inc.).

2.3 Results

2.3.1. External Gross Morphology

Mean tail length of snakes used in this study was 13.1 cm for males and 14.0 cm for females. Although absolute tail length did not differ between the sexes (Mann-Whitney Rank Sum, $U = 95.5$, $P = 0.227$), relative to snout-vent length (i.e. ratio of tail length to snout-vent length), males had significantly longer tails than females (Mann-Whitney Rank Sum, $U = 69.0$, $P = 0.027$). While some tails had no obvious external changes (Figure 2.1a, b), others had visibly distended scales on their ventral surface giving the tail a swollen, ‘puffy’ appearance which was usually more pronounced distally (Figure 2.1c, d). Tails used for histology included specimens that appeared fully intact as well as some, termed ‘stub tails’, which were clearly missing a distal portion of their length (Figure 2.1e, f). The two most extreme stub tails, one male and one female, appeared to be missing approximately 30-50% of their length (based on typical mean tail lengths for snakes in this population; Uhrig, pers. obs.). As missing tail portions would have contained parasites as well, only intact tails were
utilized for parasite counts. Tails were considered to be intact if the small conical tail tip scale was present.

2.3.2. Histopathology

*Alaria* spp. mesocercariae were present in all twelve tails examined histologically even those that did not show external morphological changes. The mesocercariae had morphology in accordance with that described by Möhl et al. (2009). General features included an oral sucker, acetabulum, penetration glands, ceca, and a tegument covered in fine spicules (Figures 2.2 and 2.3). All infections appeared to begin caudal to the cloaca with a tendency for increasing numbers of mesocercariae toward the caudal end of the tail. Histologically, the infection did not appear to differ in any consistent manner between male and female snakes and the following pathological descriptions are applicable to both sexes.

Mesocercariae were present between fascial planes and were frequently surrounded by lamellar accumulations of pale, flocculent, basophilic mucinous material (Figure 2.4a,b). These accumulations were occasionally surrounded by trabeculae or capsules of loosely organized fibrous connective tissue (granulation tissue) that was frequently invaded and expanded by mats of epithelioid macrophages, with fewer heterophils and occasional eosinophils (Figure 2.4c,d). These walled-off compartments of mucous and inflammatory cells could be described as pseudocysts (Figure 2.4a-c) since they are circular and encapsulated, but are not lined by a functional epithelium.
In most mild cases, mesocercariae tended to be confined to the fascia just ventral to the vertebral bodies that surrounded the caudal aorta and vena cava (Figure 2.5a). It was only in more severe cases in which parasites appeared to spread ventrally and laterally into the rest of the fascia, frequently ending up in a subcutaneous position with no muscle between the parasite and the deep dermis (Figure 2.5b-d).

Inflammation was not present in all animals and, in most cases, the only visible tissue damage associated with the parasites was the mass effect of the trematodes themselves, which would occasionally compress adjacent muscle bundles, producing contraction bands, loss of cross-striations, fragmented cytoplasm, and occasionally atrophy (Figure 2.6a,c). In many cases, there was obvious compression of adjacent muscles without any evidence of myodegeneration (Figure 2.6b). The majority of cases examined were characterized only by mild inflammation. In severe cases, however, tail cross sections often contained more parasites than actual host tissue: muscle bellies were completely obliterated and replaced by thin strands of fibroplasia, atrophied muscle, and inflammatory cells delineating large pseudocysts (Figures 2.5c-d, 2.6d).

2.3.3. Parasite Counts

Prevalence of Alaria spp. mesocercariae was 100% in both male and female snakes. Overall infection intensity ranged from 37 to 2286 mesocercariae in males and 11 to 2316 in females with mean values (± SE) of 966.4 (± 273.8) and 1486.5 (± 214.9) mesocercariae respectively for males and females. When considering the density of parasites per unit of tissue, mean infection density (± SE) was 66.3 (± 18.8)
mesocercariae per cm of tail for males and 98.7 (± 13.8) mesocercariae per cm of tail for females. Intersexual differences in infection intensity were not significant either before or after accounting for variation in tail length (Student’s t-tests, P > 0.100 for both).

2.4 Discussion

Our results indicate that the presence of *Alaria* spp. mesocercariae within the tails of garter snakes has notable pathological consequences with the potential to depress host fitness. Although relatively little inflammation was present in most samples, the mesocercariae appeared to have a compressive, atrophying effect on muscle fibers with the more severe cases having few or no intact muscle bellies visible. In these cases, the ventral interior of the tail had essentially become a pocket of parasites and loose fibrous connective tissue. Such extensive loss of muscle would leave only intervertebral ligaments and skin attaching the tail to the rest of the body. Weakening the tail in this manner would make it more prone to breakage. Our results, therefore, support the hypothesis of parasite mediated tail loss as suggested by La Rue (1917) and Shine et al. (1999). Our results of increasing mesocercariae in the caudal direction may also help explain observations made by Fitch (2003) in a study of tail breakage in *Thamnophis sirtalis*. Although his study made no mention of parasites, Fitch (2003) observed that some regions of the tail seemed more fragile and noted nonrandom breakage patterns with all breaks occurring caudal to the 17th percentile of tail length and many females having breaks at the 20-29th percentiles. Such regional fragility could correspond to areas of greater mesocercariae aggregation.
While garter snakes are known to autotomize their tails as a defense strategy (Cooper and Alfieri 1993; Fitch 2003; Placyk and Burghardt 2005), tail loss in not so readily employed as it is by many lizard species. Cooper and Alfieri (1993) report tail autotomy only when a snake was grasped firmly by the tail and a characteristic body rolling behavior occurred to facilitate the break. For garter snakes, tail loss is permanent and has negative fitness consequences via reduced survival and decreased mating success (Willis et al. 1982; Shine et al. 1999), thus, it seems likely that autotomy would only occur as a last resort escape strategy. With Alaria infection damaging and weakening the tail, breakage may occur in predator encounters where it otherwise would not be necessary. Additionally, if fragility is high enough, merely moving through the environment could result in tail breakage. Indeed, one of us (E.J. Uhrig) has observed a captive snake lose its tail while housed in a laboratory aquarium free from predators.

The phenomenon of parasites aggregating in an autotomizable appendage has been documented in other species such as Canarian lizards (Gallotia galloti) and spiny sand crabs (Blepharipoda occidentalis) (Matuschka and Bannert 1987; Lafferty 1999). Lafferty (1999) suggested that such aggregation would be beneficial to trophically-transmitted parasites with parasites evolving to influence the host’s tendency to lose the appendage in order to facilitate trophic transmission. Indeed, this hypothesis seems a plausible ultimate explanation for the tendency of Alaria to aggregate in the snake tails. That is, by increasing the chance of tail breakage during a predator encounter, mesocercariae increase the likelihood they will be transmitted to a subsequent host where they may be able to continue their life cycle. The propensity
for predation attempts to result in tail loss was highlighted in a study by Placyk and Burghardt (2005) who found tail loss to be positively correlated with predator diversity for *Thamnophis sirtalis sirtalis* in Michigan. For *Alaria marcianaee* and *A. mustelae*, the species identified in preliminary molecular analyses of samples obtained from red-sided garter snakes, definitive hosts include cats, raccoons, foxes, skunks, mink, and badgers all of which are potential snake predators found within the garter snakes’ range in Manitoba (Bosma 1934; Johnson 1968; Johnson 1979).

The relative lack of inflammation and eosinophilia around the mesocercariae in the snakes’ tails is somewhat surprising given that helminth infections are typically associated with such a response (Klion and Nutman 2004). However, this finding is in accordance with other studies of *Alaria* spp. (e.g., Fernandes et al. 1976; Shoop and Corkum 1984) which similarly noted an absence of inflammation. Of related interest is our observation of ‘pseudocysts’ appearing as mucus-filled compartments often containing multiple mesocercariae. While the nature of our study did not allow us to definitively determine whether the mucus was host or parasite derived, a study by Hofer and Johnson (1970) of *Alaria mustelae* mesocercarial capsules found no evidence of mucus producing cells in the parasite and concluded, therefore, that the mucus is most likely produced by the host. Regardless of the mucus’ origin, however, it is probable that the pseudocystic capsules around the mesocercariae contribute to the reduced inflammatory response. Similar immune-shielding effects of mucus production and encapsulation have been noted in other studies of parasitic infections (e.g., Riffkin et al. 1996; Theodoropoulos et al. 2001).
Although intersexual variation in various aspects of parasitic infections has been reported for a number of other species (Poulin 1996; Zuk and McKean 1996), we found no significant intersexual variation in our study. Our finding of 100% prevalence for both sexes is not surprising given that *Alaria* is acquired trophically and frogs, the parasite’s second intermediate host, are a major component of the garter snakes’ diet (Gregory and Stewart 1975). While diet composition does not differ between males and females (Gregory and Stewart 1975), we might have expected to see significant variation in infection intensity because female snakes, being larger in size than males, may consume more or larger frogs and so ingest more mesocercariae. Additionally, despite their overall larger size, the tails of females are shorter relative to body length than are male tails (Shine et al. 1999). Thus, we could reasonably expect more mesocercariae per cm of tail for females. However, despite a slight trend for females to have higher infection intensity than males, intersexual differences were not significant. Given that individual frogs can harbor thousands of mesocercariae (Fernandes et al. 1976) and a snake will eat many frogs over its lifetime, clearly not all mesocercariae consumed become permanently established in the tail. Thus, snakes may have some degree of resistance to the parasite and it is conceivable that such resistance could vary between the sexes (Zuk 2009; McClelland and Smith 2011). If females have greater resistance than males, as has been noted in a number of mammalian species (Klein 2000; Morales-Montor et al. 2004), then, despite ingesting greater numbers of parasites, fewer mesocercariae may be able to establish in female tails leading to the lack of intersexual variation in infection intensity that we observed. This explanation, however, is somewhat speculative and
clearly the topic of parasite resistance in garter snakes is one that requires further investigation.

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2.5 References


Figure 2.1. Tail morphology of male (images on the left) and female (images on the right) red-sided garter snakes (Thamnophis sirtalis parietalis). Images A and B depict the ventral surfaces of tails with no obvious external changes. Images C and D exemplify a “puffy” tail morphology where the ventral surface is swollen with distended scales particularly near the distal end of the tail. Images E and F depict a “stub” tail morphology where a portion, possibly up to 50%, of the tail is missing. Photo credit: E.J. Bentz.
Figure 2.2. Wet mount of *Alaria* sp. mesocercaria from the tail of a red-sided garter snake (*Thamnophis sirtalis parietalis*).
Figure 2.3: Images depicting two different views of *Alaria* spp. mesocercariae from the tails of red-sided garter snakes (*Thamnophis sirtalis parietalis*). Key: A. acetabulum. B. gland cells. C. cecum. D. duct of penetration gland. E. esophagus. F. penetration gland. G. oral opening with oral sucker.
Figure 2.4: Patterns of host response to mesocercariae in the tails of red-sided garter snakes (*Thamnophis sirtalis parietalis*). A. Single mesocercaria surrounded by lamellar flocculent, basophilic mucinous material (star) encapsulated in a pseudocyst formed by a layer of epithelioid macrophages and fibroblasts (arrow). B. Multifocal accretions of lamellar mucinous material occasionally mineralized at the edges. C. A pair of mesocercariae in a pseudocyst lacking mucinous material but still surrounded by a thin layer of epithelioid macrophages and fibrous connective tissue (arrow). The surrounding epimysium and perimysium of muscle bundles along with intermuscular fascia is expanded by edema fluid, heterophils, macrophages, and eosinophils. While multiple mesocercariae are observed in close association and occasionally appear to adhere to each other via acetabula, whether this is a feature of the life cycle or a simple sequela of high density within a small space is unclear. D. Multiple mesocercariae not encapsulated by pseudocysts but separated by broad trabeculae of loose fibrous connective tissue (arrow). These mesocercariae have effaced the vast majority of host muscle in this region and are present just under the dermis (star).
Figure 2.5: Potentially progressive patterns of tissue invasion in the tails of red-sided garter snakes (*Thamnophis sirtalis parietalis*). A. Mesocercariae in a relatively mild infection located just ventral to the vertebrae and surrounding large tail vessels expanding the fascia with mucus. B. Mesocercariae in a more severe infection extending through the midline fascia (arrow). C. Mesocercariae in a progressively more severe infection extending from the midline fascia ventral to the spine and spreading subcutaneously along the ventrum (arrow). D. Severe infection from a relatively cranial portion of the affected tail in which mesocercariae have massively expanded the dorsolateral subcutis (arrow).
Figure 2.6: Host tissue responses to infection in the tails of red-sided garter snakes (*Thamnophis sirtalis parietalis*). A. A combination of acute and chronic changes. Muscle bundles are separated by connective tissue expanded by edema fluid, mild fibrosis, and a mild inflammatory infiltrate composed of eosinophils, macrophages, and fewer heterophils (stars). Multifocal muscle fibers have fragmented or vacuolated cytoplasm, an acute degenerative change (arrow). B. Mesocercariae can invade fascial planes between muscle bellies with minimal inflammation and muscle damage. C. Bands of fibrous connective tissue and inflammatory cells associated with mesocercariae can surround and entrap degenerate myofibers (arrow). D. Mesocercaria surrounded by abundant inflammation composed of loose, fibroblast-rich trabeculae infused with macrophages and eosinophils as well as rafts of free epithelioid macrophages, heterophils, and eosinophils (arrow).
Chapter 3: Patterns in parasitism: interspecific and interpopulational variation in helminth assemblages and their reproductive fitness correlates in garter snakes

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Abstract:

Parasites can influence host fitness, and thus the evolution of host populations, in a variety of context-dependent ways. Investigations of the fitness and evolutionary implications of parasitism, however, first require knowledge of the parasite assemblages within hosts. Our study investigates helminths of male red-sided garter snakes (Thamnophis sirtalis parietalis) and plains garter snakes (T. radix) in Manitoba, Canada. We compare infection prevalence and density for trematodes in the lung (adult Lechriorchis sp.), visceral fat (diplostomid metacercariae), and tail tissue (Alaria spp. mesocercariae) as well as nematodes (adult Rhabdias sp.) in the lung and cestodes in the gastrointestinal tract. We assess correlations between helminth burden and snake size in both species. Further, we evaluate whether helminths predict snake body condition, testes mass, or sperm counts, all of which are crucially related to male fitness. Our results demonstrate that helminth infections are highly prevalent in both red-sided garter snake populations and in plains garter snakes. Interspecific and interpopulational differences in helminth densities, specifically members of the genera Alaria, Rhabdias, and Lechriorchis, were also apparent. Although the helminths examined in this study are all trophically transmitted, we did not find consistent correlations between helminth burden and snake size, which may indicate interpopulational and interspecific variation in
resistance or clearance of infections, or differential exposure to parasites. Helminths were significant predictors of body condition, testes mass, and sperm counts within plains garter snakes and one population of red-sided garter snakes; *Alaria* spp. and *Rhabdias* sp. densities were most frequently selected as predictors, thus, these helminths may be particularly influential on host fitness. However, there are many ways in which parasites can influence host fitness beyond altering body condition, gonads, or gametes. Thus, while our results provide evidence for fitness implications of helminth infections in garter snakes, future investigations are needed to further elucidate the fitness consequences of parasitism in these species.

### 3.1 Introduction

Parasites strongly influence host evolution via both natural and sexual selection (Lafferty 1993; Able 1996; Poulin and Vickery 1996; Penn and Potts 1998; Boots and Haraguchi 1999; Poulin and Thomas 1999; Duffy and Sivars-Becker 2007). The nature of the host-parasite relationship is complex and the effects of parasitic infections on individual host fitness as well as on the evolution of host populations are extremely varied and dependent upon a myriad of interacting factors, including the physiologies and life histories of both host and parasite, the timing and intensity of the infection, as well as ecological factors such as resource availability, predation risk, and the presence of other parasite species (Price et al. 1986; Forbes 1993; Hatcher et al. 2006; French et al. 2007; Lagrue and Poulin 2007; Martin et al. 2008; Stjernman et al. 2008). Although many studies of the effects of parasitism focus on only a single parasite species, interspecific interactions and competition
occurring among parasites within the host have important implications for host fitness (Graham et al. 2005; Telfer et al. 2010; Johnson and Hoverman 2012; Gorsich et al. 2014). Therefore, to assess the effects of parasitism on host fitness, it is crucial to understand the assemblage of parasites commonly infecting the host species.

Parasites, by the generally accepted definition, have adverse effects on host fitness. These effects occur via alterations of host survival or more subtly through influencing host reproduction; in either case, the lifetime production of offspring is curtailed in some way. The effects of parasites on reproductive fitness can occur, for example, via alterations of gametogenesis, reproductive behaviors and/or sexual signaling (e.g. Hamilton and Zuk 1982; Read 1990; Forbes 1993; Lafferty and Kuris 2009). More general effects of parasites on host body condition can also have reproductive implications as individuals in poorer body condition may forego attempts at reproduction, have reduced mating success, or produce fewer offspring (e.g. Naulleau and Bonnet 1996; Dyrcz et al. 2005; Shine and Mason 2005). In some instances, the presence of parasites exerts direct effects on host reproductive fitness. For example, parasites that reside in the gonads often directly damage gonadal tissue possibly leading to castration (Baudoin 1975; Lafferty and Kuris 2009). However, more indirect effects are also possible as parasites may commandeer host resources that could otherwise be allocated to reproduction (Forbes 1993; Hurd 2001; Lafferty and Kuris 2009). Additionally, it may not be the effect of parasites per se that leads to alterations of host reproduction, but rather activation of the host’s immune system may divert resources away from reproduction and toward the immunological response to the parasite (Folstad and Karter 1992; Sheldon and Verhulst 1996; Schmid-Hempel
Thus, there is a complex interplay between parasitism and host physiology and the mechanisms via which parasitic infections alter host reproductive fitness may not be immediately apparent.

Among vertebrate taxa, there is a relative paucity of studies focusing on parasitism and its reproductive implications in reptiles, and snakes in particular. However, at least one snake genus, *Thamnophis*, is an ideal candidate for such studies. With more than 30 species ranging throughout North America (Rossman et al. 1996), *Thamnophis* lends itself well to comparative studies of parasitism and host life history, and could potentially provide insight into host-parasite coevolution. *Thamnophis* species, and particularly the red-sided garter snake (*T. sirtalis parietalis*), have been the focus of numerous behavioral and physiological studies (e.g. Crews et al. 1984; Krohmer et al. 1987; Mason et al. 1989; Mason 1993; LeMaster and Mason 2002; Shine et al. 2003; O’Donnell et al. 2004; Shine and Mason 2005; Lutterschmidt and Mason 2008; Uhrig et al. 2012; Friesen et al. 2013). In contrast, parasites of *Thamnophis* have received relatively little attention in the literature beyond simple mention of their presence (e.g., Gibson and Rabalais 1973; Rau and Gordon 1978; Goldberg and Bursey 2002; Jiménez-Ruiz et al. 2002). With reproduction so extensively studied in garter snakes, we are well placed to begin investigating the reproductive implications of parasitism in these snakes.

During the breeding season that follows emergence from winter hibernation, red-sided garter snakes form large mating aggregations (~35,000 snakes at a single den site; Shine et al. 2006) that include “mating balls” wherein a single female is surrounded by multiple males vigorously courting her and vying for position to mate.
Male courtship is energetically expensive and male red-sided garter snakes in better body condition generally have more mating opportunities as they are able to remain in mating aggregations for longer periods of time before migrating to feeding grounds (Shine and Mason 2005). Plains garter snakes (*T. radix*) also form mating aggregations although in much smaller numbers compared to red-sided garter snakes (Gregory 1977; Rossman et al. 1996). Female garter snakes can mate with more than one male during the breeding season and so sperm competition is likely to occur in these snakes (Gibson and Falls 1979; Schwartz et al. 1989; McCracken et al. 1999; Friesen et al. 2014a,b). In the context of sperm competition, having greater sperm numbers, which is often positively associated with testes size, can increase a male’s likelihood of successfully siring offspring with a given female (Parker 1990; Stockley et al. 1997; Parker and Pizzari 2010). Thus, any effects of parasites on male body condition, testes mass, or sperm counts would have clear implications for male reproductive fitness.

In the current study, we investigate interspecific and interpopulational variation in the helminth parasites of male garter snakes in Manitoba, Canada in order to provide a basis for additional studies of host-parasite interactions. As a starting point, we focus on the most commonly observed helminths in these snakes, namely adult trematodes of the lung, metacercariae in the visceral fat, and mesocercariae in the tail; adult nematodes of the lung, and gastrointestinal cestodes. To determine parasite burden, we dissected males from two allopatric populations of red-sided garter snakes and one population of plains garter snakes. We compare parasite prevalence and density between species and populations. We also assess whether
helminth burden is correlated with snake size. To evaluate whether helminth burden affects reproductive fitness, we use model selection to determine if helminth burden predicts body condition, testes mass, or sperm counts as these attributes are all crucial to a male reproductive success. Our findings provide a useful basis for future investigations of the effects of parasitism in these snakes.

3.2 Methods

3.2.1. Animal Collection

All snakes utilized in this study were wild caught individuals collected by hand during the breeding season (late April, early May) following their emergence from winter hibernation in Manitoba, Canada. In 2012, red-sided garter snakes (*Thamnophis sirtalis parietalis*) were collected from two den sites (Figure 3.1): one in the Interlake region north of the town of Inwood (50°31′58″N; 97°29′71″W; N = 21 males) and another on Snake Island in Lake Winnipegosis (51° 38′38″N 99° 49′25″W; N = 23 males); these two populations will be referred to as IW and SI, respectively. Snakes were transported to Oregon State University and housed in 10-gallon aquaria (6 snakes per tank) within light and temperature controlled environmental chambers (13:11 hr, L:D; 24:18°C) until dissection in September 2012. Snakes were provided water *ad libitum* and fed weekly with either fresh chopped earthworms or thawed from frozen salmon fry.

In 2013, all red-sided garter snakes (N = 60 males) were collected during the breeding season from the Inwood den site (IW population) and plains garter snakes (*Thamnophis radix*; N = 24) were collected from a location southeast of West Shoal
Lake in Manitoba (50°15'21”N; 97°34’30”W; Figure 3.1). Snakes collected in 2013 were transported (approximately 77 km) to the Chatfield Research Station where they were kept in seminatural outdoor enclosures (1m x 1m x 1m nylon construction as in Moore and Mason 2001) until being dissected; snakes were provided with water ad libitum, but were not provided food as garter snakes are generally aphagous during the breeding season (O’Donnell et al. 2004). All dissections were conducted at the research station within three weeks of animal collection.

3.2.2. Dissections and Parasite Identification

Following euthanasia with an overdose of Brevital® sodium (methohexital), lateral incisions were made along each side of a snake’s ventral surface from the cloaca to just cranial to the heart. The skin of the ventral surface was then reflected to expose the viscera. Any remaining mesenteric tissue was carefully removed with forceps. Gonads and visceral fat bodies were removed and weighed (± 0.001 g) individually on a digital scale (A&D Fx-300i balance).

In preliminary dissections, we consistently found helminth parasites to be located primarily in the lung, gut, visceral fat, and tail; these locations, therefore, were the focus of subsequent dissections in which we evaluated parasite prevalence (percentage of infected individuals), and where possible, the density of infection (number of parasites per unit of tissue) (definitions as in Bush et al. 1997). Helminths were identified via microscopy and referral to relevant published morphological descriptions (e.g., Price 1936; Byrd and Denton 1938; Chandler 1942; Baker 1978; Möhl et al. 2009). Additionally, samples of parasites from the lung, tail, and fat of
red-sided garter snakes were sent to Dr. Michael Kinsella (HelmWest Laboratory, Missoula, Montana), lung and tail parasites were also sent to Dr. William Font (Southeastern Louisiana University, Hammond, Louisiana) for confirmation of our identifications. As part of a separate study, tail parasites were also sent to Dr. Vasyl Tkach (University of North Dakota, Grand Forks, North Dakota) for molecular analyses.

**Lung Parasites:**

The lung was removed by cutting the trachea close to the lung’s cranial end and then gently separating the lung from the rest of the body with forceps. In garter snakes, a single lung is present (the second being vestigial) and consists of an anterior vascularized region and a posterior non-vascularized region known as the saccular lung (Wallach 1998; Shine et al. 2003); during dissection, the distinction between the vascular and saccular lung regions is readily observable even without the aid of a microscope. A cut was made to separate the two regions of the lung and the anterior region was weighed on the digital scale; we did not attempt to weigh the saccular lung as, due to its very thin and easily torn tissue, it was not possible to cleanly remove the surrounding mesentery and the region does not contribute greatly to total lung mass. Both regions of the lung were examined for adult trematodes (identified as *Lechriorchis* sp.) which are relatively large, easily recognizable and found primarily in the saccular lung although they were sometimes present in the anterior lung as well. If present, these trematodes were removed with forceps and counted
immediately; density was considered to be the number of adult trematodes per gram of lung tissue.

The anterior lung, in which adult nematodes (identified as *Rhabdias* sp.) occurred, was placed in a 30 ml glass vial of distilled water (5 ml) and allowed to soak overnight to facilitate the exit of nematodes from the lung tissue (similar to Waldrup et al. 1998). The following day, 5 ml of 10% formalin was added to the vial to achieve a final concentration of 5% formalin in order to preserve the tissue and associated parasites until later counting. For parasite counts, the lung tissue and the accompanying formalin solution were transferred to a gridded Petri dish under a stereomicroscope (Nikon SMZ-745T). The lung was examined for the presence of any nematodes remaining lodged in the tissue and the dish was methodically scanned for nematodes. A tally counter was used to keep track of parasite counts. Density was considered to be the number of adult nematodes per gram of lung tissue.

**Visceral Fat Parasites:**

The parasites contained within the fat were encysted larval trematodes (metacercariae tentatively identified as *Fibricola* sp.). Using fine forceps, the visceral fat body was separated from surrounding tissue and weighed on a digital scale. In order to separate the encysted metacercariae from the tissue, the fat was placed in a 30 ml beaker and 15 ml of distilled water was added. A Tissue Tearor™ homogenizer was then used to homogenize the fat to facilitate rupturing of the metacercarial cysts (similar to Shoop 1989). The sample was then left overnight to allow the metacercariae to settle to the bottom of the vial. Once the metacercariae had settled,
tissue debris and excess liquid was pipetted from the surface of the solution leaving 5 ml remaining in the vial; to this volume, 5 ml of 10% formalin was added to preserve the parasites. For parasite counting, the contents of the vial were transferred to a Bogorov-type plankton counting chamber under a stereomicroscope (Nikon SMZ-745T); a tally counter was used to keep track of parasite counts. Parasite density was considered to be the number of metacercariae per gram of fat.

**Tail Parasites:**

The tail was severed 1 cm caudal to the cloaca, slit open along its ventral surface, and then placed in a vial of 5% formalin of sufficient volume to cover the tissue so that it was preserved for later counts of larval trematodes (identified as the mesocercarial stage of *Alaria* spp.). For parasite counting, the contents of the vial were transferred to a Petri dish. Using a stereomicroscope (Nikon SMZ-745T), the trematodes were separated from the tail tissue using fine forceps. The mesocercariae were then transferred to a Bogorov-type plankton counting chamber where they were counted under the same stereomicroscope. A tally counter was used to keep track of parasite counts. Parasite density was considered to be the number of mesocercariae per cm of tail tissue.

**Cestodes:**

The alimentary canal was cut just cranial to the cloaca and inspected for the presence of cestodes (tapeworms) by squeezing the gut with forceps, which were then moved along its length in the caudal direction. If present, the worms were extruded
from the caudal end of the gut. Due to the difficulty of removing cestodes fully intact, we did not attempt to count these parasites and noted only their presence or absence.

3.2.3. Sperm Counts:

For male snakes, the ductus deferentia were cut at their posterior ends just cranial to the cloaca and at the anterior ends where they joined the testes. The ducts were then carefully removed with fine forceps and placed in a 1.5 ml microcentrifuge tube containing 1 ml of sperm washing medium (Irvine Scientific) until the rest of the dissection was complete. Both ductus deferentia and medium were then transferred to a small Petri dish containing an additional 2 ml of sperm washing medium. Under a stereomicroscope (Nikon SMZ-745T), sperm were extruded from each ductus deferens by holding the duct with a pair of fine forceps and squeezing with a second pair of forceps which were moved along the duct’s length. Using this technique, masses of sperm were readily visible extruding from the open end of the duct. The process of squeezing and moving the forceps along the ductus deferens was repeated until no more sperm could be seen exiting the duct. The duct tissue was then discarded and the solution of sperm and sperm washing medium were transferred to a 4 ml culture tube and centrifuged. The resulting pellet of sperm was collected in a volume of 1 ml of sperm washing medium and transferred to a microcentrifuge tube which was gently shaken to homogenize the solution. Sperm counts were conducted using the methods described by Friesen et al. (2013). Briefly, a 4 ul aliquot of the sample was examined using a Petroff-Hauser counting chamber at 10x magnification on a phase contrast compound microscope (Olympus CX31) equipped with an
Olympus DP-21 digital camera and CellSens software (Olympus). Overly concentrated samples (>100 sperm on the counting grid) were diluted with additional sperm washing medium. Once a suitable concentration was achieved, a photo of the sample on the counting grid was taken. The process was repeated for a total of three replicates per sperm sample. The resulting photos were saved as JPEG files for later counting. Sperm counts of the JPEG images were conducted using the CellSens software.

3.2.4. Statistical Analyses

We used residuals from linear regressions of ln mass on ln snout-vent length (SVL) to estimate body condition (Shine and Mason 2005); separate regressions were done for each population and species. Male mass, snout-vent length (SVL), tail length, and body condition were compared between populations and species using either t-tests or Mann-Whitney Rank Sum tests. Comparisons of parasite prevalence (% of individuals infected) were made using Chi-square tests or Fisher exact tests. As our dissections focused on specific organs and tissues, we chose to compare parasite densities (no. of parasites per unit of tissue) rather than intensities (no. of parasites per infected individual). We used Spearman rank correlations to evaluate the relationships between snake SVL and the total number of each helminth. All of the aforementioned analyses were conducted in either SigmaPlot (version 12.5, Systat Software, Inc.) or XLStat (version 2013.4.8, Addinsoft).

In order to assess potential fitness effects of parasite infections, we used a multiple regression approach. Specifically, we used forward stepwise regression
[‘step’ function with AIC criteria from the MASS package of R (Venables and Ripley 2002)] to explore whether parasite densities predicted snake body condition, testes mass, or sperm counts. For testes mass, we used standard residuals from regressions of ln testes mass on ln SVL. Similarly, for sperm we used standard residuals from a regression of square root transformed sperm counts on ln SVL. We set the lower limit for the stepwise procedure as the intercept only model and the upper limit as a full model containing square root transformed densities of Lechriorchis sp., Rhabdias sp., Alaria sp., and VF metacercariae. For models of body condition, ln visceral fat mass was also included as a predictor and, for models of testes and sperm, body condition was included as a predictor. In instances where the stepwise procedure selected a model containing an interaction term that was nonsignificant, we removed the interaction and checked the AIC of the resulting simpler model; if the AIC increase was small (< 2 units) then we chose the simpler model as our final model (Burnham and Anderson 2002). Stepwise regression was carried out in version 2.15.2 of the R statistical program (R Core Team 2012).

3.3 Results

3.3.1. Parasite Identification:

Adult nematodes of the genus Rhabdias were identified in the anterior region of the lung where they were generally embedded within the tissue (Figure 3.2a). The identification of Rhabdias sp. was based on morphological characteristics which were consistent with those described by Baker (1978); the small size of the nematodes (approximately 2.5 mm), their esophagus length (240 μm) and reduced buccal cavity
are suggestive of *R. fuscovenosa* (M. Kinsella, pers. comm.), which previous studies have found in the lungs of *T. sirtalis* (Gibson and Rabalais 1973; Baker 1978), but additional analyses are needed to confirm the species-level identification.

Adult trematodes of the genus *Lechriorchis*, frequently laden with eggs, were sometimes found in both regions of the lung, but occurred mainly in the saccular region (Figure 3.2b). The genus-level identification of *Lechriorchis* is based on morphological characteristics consistent with Byrd and Denton (1938); M. Kinsella and W. Font offered confirmation of the genus identification. *Lechriorchis* spp., specifically *L. primus*, have previously been observed in the lungs of *T. sirtalis* (Rau and Gordon 1978, 1980).

Parasites encysted in the visceral fat were identified as encysted larval (metacercarial stage) diplostomid trematodes, which, based on morphological characteristics consistent with descriptions provided by Chandler (1942) and Seo (1990), may belong to the genus *Fibricola* (M. Kinsella, pers. comm.; Figure 3.2c). *Fibricola* spp. have been previously observed in the visceral fat of garter snakes (Cuckler 1940; Turner 1958); however, this genus-level identification is tentative and we will, therefore, refer to these trematodes as visceral fat metacercariae hereafter (abbrev. VF metacercariae).

Parasites of the tail were identified as unencysted larval (mesocercarial stage) trematodes of the genus *Alaria* based on morphology consistent with Möhl et al. (2009). This genus-level identification was confirmed by W. Font and M. Kinsella. Subsequent molecular analyses further confirmed the *Alaria* identification and indicated the presence of at least three *Alaria* species including *A. mustelae*, *A.*
marcianae, and another unidentified species (pers. comm. V. Tkach; Figure 3.2d).

Both *A. mustelae, A. marcianae* have been previously noted to infect garter snakes (e.g., Rau and Gordon 1980; Möhl et al. 2009).

The gastrointestinal parasite that we observed was a cestode (tapeworm) which we were unable to classify further as we did not successfully locate a scolex in any of the samples.

3.3.2 Interpopulational Comparisons: Inwood versus Snake Island Populations:

Neither mass nor body condition of male snakes differed between the IW and SI populations (mass: Student’s t-test, \(t_{42} = -0.028, P = 0.978\); body condition: Mann-Whitney Rank Sum, \(U = 234.00, P = 0.869\)). However, males of the SI population had significantly greater snout-vent length (SVL: Student’s t-test, \(t_{42} = 2.797, P = 0.008\)) and had longer tails (Mann-Whitney Rank Sum; \(U = 102.500, P = 0.001\)).

**Parasite Assemblages:**

All of the helminth parasites examined (*Rhabdias, Lechriorchis, Alaria, VF metacercariae, and cestodes*) were found in both populations. Based on Spearman rank correlations, within the IW population, no significant correlations were found between snake SVL and total numbers of any helminth (*Rhabdias*; \(\rho = 0.356, P = 0.111\); *Lechriorchis*; \(\rho = 0.064, P = 0.780\); *Alaria*; \(\rho = 0.393, P = 0.085\); VF metacercariae; \(\rho = -0.006, P = 0.975\)).

Within the SI population, no significant correlations were found between snake SVL and total numbers of lung helminths (*Rhabdias*; \(\rho = -0.027, P =\)
Lechriorchis: \( \rho = 0.044, P = 0.837 \), but both Alaria (\( \rho = 0.574, P = 0.005 \)) and VF metacercariae (\( \rho = 0.516, P = 0.012 \)) were positively correlated with SVL.

None of the helminths differed significantly in prevalence between the two populations (Fisher exact tests; \( P < 0.05 \) for all; Tables 3.1 and 3.2). However, the SI population had significantly higher densities of both lung parasites with more than twice as many Rhabdias sp. and three times as many Lechriorchis sp. per gram of lung tissue compared to the IW population (Table 3.1). The density of VF metacercariae did not differ between the populations (Mann-Whitney Rank sum, \( U = 219.0, P = 0.605 \)), but the density of Alaria spp. was significantly higher in the IW population which had nearly twice as many mesocercariae per cm of tail tissue compared to the SI population (Mann-Whitney Rank sum, \( U = 122.0, P = 0.014 \), Table 3.2).

**Fitness Correlates:**

Using forward stepwise regression with separate analyses for each population, we evaluated whether there was evidence for helminth densities as predictors of snake body condition, testes mass, or sperm counts. For the SI population, model selection revealed Alaria spp. density as a significant predictor of body condition, while Alaria spp. and Rhabdias sp. were both significant predictors for testes mass, and Alaria spp., Rhabdias sp., and Lechriorchis sp. were significant predictors in the model of sperm counts (Table 3.5). With regard to the model of sperm counts for the SI population,
all predictors in the final model were parasite densities and the model explains more than 40% of the variation in sperm counts (adj. $R^2 = 0.418$). For the IW population, parasite densities appeared to be less important for predicting the dependent variables. No helminth densities were included as predictors in the final model of testes mass for the IW population. *Rhabdias* sp. and *Lechriorchis* sp. were included as predictors in the final models for body condition and sperm counts, respectively, but neither parasite was a significant predictor (Table 3.5). Indeed, for sperm counts, the overall final model itself was not significant (Adj. $R^2 = 0.089$, $P = 0.108$; Table 3.5).

3.3.3. Interspecific Comparison: red-sided garter snakes and plains garter snakes:

While snout-vent length and body condition did not differ between species (SVL: $U = 544.00$, $P = 0.082$; body condition: $U = 705.00$, $P = 0.886$), male plains garter snakes (*T. radix*) had significantly greater body mass and longer tails compared to male red-sided garter snakes (*T. s. parietalis*) from the IW population (Mann-Whitney Rank Sum tests, mass: $U = 201.500$, $P < 0.001$; tail length: $U = 453.50$, $P = 0.011$).

*Parasite Assemblages:*

*Rhabdias* sp., *Lechriorchis* sp., *Alaria* spp., and VF metacercariae were present in both snake species, but cestodes were only found in red-sided garter snakes. Based on Spearman rank correlations, for red-sided garter snakes, significant positive correlations were found between snake SVL and total numbers of *Rhabdias* sp. ($\rho = 0.500$, $P < 0.001$), *Lechriorchis* sp. ($\rho =
0.290, P = 0.025), *Alaria* spp. (ρ = 0.491, P < 0.001) and VF metacercariae (ρ = 0.459, P = 0.036). For plains garter snakes, total number of *Alaria* spp. had a significant positive correlation with SVL (ρ = 0.538, P = 0.007), but no significant correlations were found with the other parasites (*Rhabdias* sp.: ρ = 0.091, P = 0.676; *Lechriorchis* sp.: ρ = 0.024, P = 0.911; VF metacercariae: ρ = 0.248, P = 0.238). In red-sided garter snakes, the total number of *Alaria* spp. mesocercariae was also positively correlated with tail length (ρ = 0.318, P = 0.015), but no such significant correlation was found for plains garter snakes (ρ = 0.214, P = 0.311).

The prevalence of cestode infections was significantly greater for red-sided garter snakes (21.7% of individuals were infected) whereas no plains garter snakes were infected. The prevalence of *Lechriorchis* sp. was also greater for red-sided garter snakes with 71.7% of individuals being infected compared to only 45.8% of plains garter snakes. None of the other parasites differed in prevalence between the two snake species (P > 0.05 in all cases, Tables 3.3, 3.4). Both lung parasites occurred at significantly higher densities in red-sided garter snakes with *Rhabdias* sp. density being nearly twice as high and *Lechriorchis* sp. density being over 4.5 times higher in red-sided garter snakes compared to plains garter snakes (Table 3.3). In contrast, *Alaria* spp. density was more than two times greater in plains garter snakes than in red-sided garter snakes. The density of VF metacercariae did not differ between species (Mann-Whitney Rank Sum, U = 185.0, P = 0.130).
Fitness Correlates:

Using forward stepwise regression with separate analyses for each species, we evaluated whether there was evidence for parasite densities as predictors of snake body condition, testes mass, or sperm counts. For plains garter snakes, *Lechriorchis* and VF metacercariae were included in the final model of body condition, but only the density of VF metacercariae was actually a significant predictor of body condition. For testes mass in plains garter snakes, *Lechriorchis* sp. and *Alaria* spp. were included in the final model, but only *Alaria* spp. density was a significant predictor (Table 3.6); this model, which contained only parasite densities as predictors, explained 47% of the variation in testes mass for plains garter snakes (adj. $R^2 = 0.470$). *Rhabdias* sp. density was revealed as a significant predictor of plains garter snake sperm counts. With regard to red-sided garter snakes, intercept only models were selected as the final models for body condition and testes mass (Table 3.6). *Rhabdias* sp. and VF metacercariae were included in the final model for sperm counts in red-sided garter snakes; however, neither parasite was a significant predictor (Table 3.6).

3.4 Discussion

The results of our study demonstrate that helminth infections are highly prevalent and generally of high density in garter snakes in Manitoba, Canada. We found red-sided garter snakes of two distinct populations to be commonly infected with adult *Rhabdias* sp. nematodes and adult *Lechriorchis* sp. trematodes in their lung
tissue, diplostomid metacercariae in their visceral fat bodies, mesocercariae of *Alaria* spp. in their tail tissue, and gastrointestinal cestodes. With the exception of the cestodes, all of the aforementioned helminths were also found in the population of plains garter snakes that we examined. The helminth assemblages in these snakes exhibit both interspecific and interpopulational variation most notably in terms of parasite density. Helminth densities predict male attributes that have clear relevance to reproductive fitness, namely body condition, testes mass, and sperm counts.

At least one type of helminth was a significant predictor of body condition, testes mass, and sperm counts for the Snake Island (SI) population of red-sided garter snakes and for plains garter snakes. No interaction terms were selected as significant predictors of any of the dependent variables although, at least for the SI population, testes mass and sperm counts were predicted by the additive effects of two or more types of helminths. Of all the helminths in this study, *Alaria* spp. density was most often included as a significant predictor as it predicted body condition, testes mass, and sperm counts in the SI population of red-sided garter snakes as well as testes mass in plains garter snakes. *Rhabdias* sp. density was also a predictor of testes mass for the SI population and a predictor of sperm counts in both the SI population and plains garter snakes. *Lechriorchis* sp. was only selected as a predictor of sperm counts in SI snakes, and VF metacercariae were only a predictor of body condition in plains garter snakes. Thus, *Alaria* spp. and *Rhabdias* sp. may be the most likely helminth infections to influence male garter snake reproductive fitness, although their influences may not be uniform across species or populations. Indeed, for the Inwood (IW) population of red-sided garter snakes in both 2012 and 2013, helminth densities
were not significant predictors in regressions of any of the dependent variables. Thus, for the IW population, despite having quite high density infections, the male fitness correlates included in the current study do not appear to be significantly affected by the helminth parasites we examined. The reasons for the apparent interpopulational difference in the predictiveness of helminth density for male fitness correlates is not clear, but may be related to different population sizes as Inwood is a much larger population (~35,000 snakes; Shine et al. 2006) than Snake Island (estimated at <500 snakes; Mason et al. 1991), therefore, selection, including parasite-mediated selection, may be acting differently in the two populations (Frankham 1996; Gandon and Michalakis 2002; Charlesworth 2009).

In light of the aforementioned findings, it is also important to recognize that even where we found no evidence of our focal helminths affecting body condition, testes mass, and sperm counts, they may nonetheless influence male fitness in other ways not examined in the current study (e.g. changes in reproductive behaviors, mating success). *Alaria* spp. mesocercariae, for example, aggregate in the snakes’ tail tissues where their presence damages muscle fibers weakening the tail and increasing the likelihood of tail loss (Uhrig et al, chapter 2 of this dissertation). The loss of the tail in turn has marked fitness consequences for males by impeding their competitive ability and reducing mating success (Shine et al. 1999). Infections of *Rhabdias* sp. nematodes within the lungs of anurans adversely affect host growth, survival, and locomotory endurance (Goater and Ward 1992; Kelehear et al. 2009); given the high densities of *Rhabdias* sp. found within the vascular portion of the lung in the current study, it is plausible that *Rhabdias* sp. could similarly affect garter snakes,
particularly with regard to courtship vigor and endurance, yet this remains to be investigated. Potential fitness implications of *Lechriorchis* in the lung and diplostomid trematodes present in visceral fat (putatively *Fibricola* sp.) have also not been thoroughly explored.

In terms of parasite assemblages, prevalence of all helminths was similar between red-sided garter snake populations while plains garter snakes had lower prevalence of *Lechriorchis* and zero prevalence for cestodes. Densities of *Alaria* and both lung helminths varied between populations and species. All helminths in our study can be trophically acquired by garter snakes, mainly via the consumption of infected amphibians (Talbot 1933; Turner 1958; Möhl et al. 2009; Langford and Janovy Jr 2009). As we collected all snakes before they were likely to have begun feeding in the spring, and the snakes subsequently kept in the laboratory (i.e. those used for interpopulational comparisons) were not fed a diet from which they were likely to acquire the focal helminths, then the parasites we observed represent those that overwintered within their host snakes. This finding is in accordance with Rau and Gordon (1978) who observed helminths, including members of the genera *Rhabdias*, *Lechriorchis*, and *Alaria*, overwintering in *Thamnophis sirtalis sirtalis* in Quebec, Canada.

Interestingly, our results are somewhat mixed with regard to correlations between snake size (snout-vent length) and helminth burden. Given that our focal helminths are trophically transmitted to the snakes, we generally would have expected positive correlations between snake size and total numbers of each helminth because larger snakes would be likely to consume more and/or larger prey and so accumulate
greater numbers of parasites (Arneberg et al. 1998; Vitone et al. 2004). For plains garter snakes, however, only the number of *Alaria* spp. mesocercariae was positively correlated with snake size. The reason for the lack of significant correlations between size and the other helminths we examined in this species is unclear, but could reflect the snakes’ resistance to the parasites (e.g. Coltman et al. 2001; Carton, et al. 2005; Anthony et al. 2007). If plains garter snakes have some resistance to infection by *Rhabdias* sp., *Lechriorchis* sp., and VF metacercariae, then fewer of these parasites may establish within the host and correlations with size may not be observed. On average, plains garter snakes tend to be larger bodied compared to red-sided garter snakes and so might be expected to ingest more parasites; we might then expect plains garter snakes to have greater parasite densities than red-sided garter snakes, but this was only the case for *Alaria*; both lung parasites were actually present in greater density within red-sided garter snakes, and *Lechriorchis* also had greater prevalence in this species. Thus, plains garter snakes and red-sided garter snakes may differ in their resistance to at least some helminth parasites; a similar argument can be made for different populations of red-sided garter snakes as we discuss below.

For red-sided garter snakes from the Inwood (IW) population in 2012, there were no significant correlations between snake size and helminth numbers while, for the same population in 2013, total numbers of all helminths were positively correlated with snake size. As the snakes in 2012 were kept in the laboratory for approximately 3 months prior to dissection and were not likely acquiring any additional helminths during this time, it may be that snakes were able to clear some parasites from their system (e.g. LaFonte and Johnson 2013), thus, reducing or eliminating correlations
with size. However, snakes from the Snake Island (SI) population were kept in the laboratory for the same period of time in 2012 and still had positive correlations between SVL and numbers of *Alaria* spp. or VF metacercariae although no such correlations were found for lung parasites (*Rhabdias* sp. and *Lechriorchis* sp.). These results could indicate interpopulational differences in ability to clear certain helminths. That is, compared to IW snakes, SI snakes may have been less able to clear lung helminths and so the correlation between SVL and numbers of these parasites remained evident; indeed, SI snakes also had greater densities of both lung helminths compared to IW snakes. However, the idea of variation in parasite clearance remains speculative as we do not have parasite data for both populations prior to their being held in captivity and it may be that the SI population simply had more lung helminths to begin with. However, greater initial numbers of parasites in the SI snakes would not exclude the possibility of interpopulational variation in resistance as SI snakes could be more susceptible to the establishment of lung parasites they take in trophically. Interestingly, in both interspecific and interpopulational comparisons it is the patterns of lung helminths that suggest differential parasite resistance, thus, there may be more variability in how the immune system interacts with these parasites.

The observed differences in helminth prevalences and densities need not arise from variation in host immunological responses. Rather, differences in diet composition between species and populations may lead to variation in parasite burdens (e.g. Reimchen and Nosil 2001; Lagrue et al. 2011). For example, although diets of both red-sided and plains garter snakes consist largely of frogs (Gregory and
Stewart 1975; Tuttle and Gregory 2009), which are intermediate hosts for at least some helminths in our current study, plains garter snakes regularly include small mammals in their diet (Tuttle and Gregory 2009). Therefore, if plains garter snakes reduce frog consumption in favor of mammalian prey, this could lead to lower accumulation of the helminths we have examined. Similarly, disparate populations of garter snakes may also differ in diet composition simply due to prey availability. Kephart (1982) found geographical variation in diet for *T. sirtalis* in California at sites in much closer proximity to one another than are the populations in our study. Additionally, frog species that are common prey of garter snakes in Manitoba (e.g. wood frog, *Rana (Lithobates) sylvatica*, and chorus frog, *Pseudacris triseriata*; Gregory and Stewart 1975) may differ in their own parasite burdens such that snake populations consuming multiple frog species in varying proportions will acquire different numbers of parasites.

Our study is, to the best of our knowledge, the first to provide interpopulational and interspecific comparisons of helminth infections in garter snakes and to provide evidence for a link between helminth densities and garter snake reproductive fitness. Thus, our results verify the importance of considering parasites as selective forces acting within garter snake populations. Future studies are needed to explore the possibility of host variation in parasite resistance underlying the observed patterns in helminth assemblages, to further clarify the role of helminths in influencing snake reproductive fitness, and to elucidate the mechanisms underlying the parasite-mediated fitness consequences. Additionally, as our study focused only on males, studies of female snakes would also be informative as it is not uncommon
for parasite burdens and their associated fitness implications to vary between the sexes (Folstad and Karter 1992; Poulin 1996; Zuk and McKean 1996; Zuk 2009).

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3.5 References:


Figure 3.1. Map of Manitoba, Canada depicting garter snake study populations. Star symbols denote the two populations of red-sided garter snake (*Thamnophis sirtalis parietalis*) with the Snake Island population (purple star) approximately 250 km northwest of the Inwood population (red star). The plains garter snake (*T. radix*) population (black triangle) is approximately 35 km southwest of the Inwood population.
Figure 3.2. Helminth parasites from red-sided garter snakes (*Thamnophis sirtalis parietalis*) in Manitoba, Canada. A) Adult nematode, *Rhabdias* sp., from the anterior lung, B) Adult trematode, *Lechriorchis* sp., containing a mass of eggs, from the saccular lung, C) diplostomid metacerceria, possibly *Fibricola* sp., from the visceral fat, D) mesocercaria of *Alaria* sp. from the snake’s tail.
Table 3.1. Prevalence, density, and range for parasites found in the lung of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) from two populations in Manitoba, Canada. Densities are shown as mean values (± SE) and median values (25th, 75th percentiles).

<table>
<thead>
<tr>
<th>Population</th>
<th>Prevalence</th>
<th>Density (No. per g lung)</th>
<th>Range (No. per g lung)</th>
<th>Prevalence</th>
<th>Density (No. per g lung)</th>
<th>Range (No. per g lung)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean: 33.3 (± 5.5)</td>
<td>4.1-90.9</td>
<td>66.7%</td>
<td>Mean: 17.5 (± 1.9)</td>
<td>0-80.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median: 26.7 (17.2, 51.7)</td>
<td></td>
<td></td>
<td>Median: 5.5 (0.0, 35.3)</td>
<td></td>
</tr>
<tr>
<td>Inwood</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snake Island</td>
<td>100%</td>
<td>Mean: 77.1 (± 8.2)</td>
<td>34.7-159.7</td>
<td>87.0%</td>
<td>Mean: 55.4 (± 13.7)</td>
<td>0-257.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median: 64.3 (49.4, 98.5)</td>
<td></td>
<td></td>
<td>Median: 27.1 (9.9, 78.5)</td>
<td></td>
</tr>
<tr>
<td>Fisher Exact</td>
<td></td>
<td></td>
<td></td>
<td>Fisher Exact</td>
<td></td>
<td></td>
</tr>
<tr>
<td>test</td>
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<td></td>
<td>test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P = 1.00</td>
<td></td>
<td></td>
<td></td>
<td>P = 0.155</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(IW: N = 21,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SI: N = 23)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rank Sum</td>
<td></td>
<td>U = 71.50</td>
<td></td>
<td>Rank sum</td>
<td>U = 142.5</td>
<td></td>
</tr>
<tr>
<td>P &lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P = 0.020</td>
<td></td>
</tr>
<tr>
<td>(IW: N = 21,</td>
<td></td>
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<tr>
<td>SI: N = 23)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2. Prevalence, density, and range for parasites found in the tail, visceral fat, and gastrointestinal tract of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) from two populations in Manitoba, Canada. Densities are shown as mean values (± SE) and median values (25<sup>th</sup>, 75<sup>th</sup> percentiles).

<table>
<thead>
<tr>
<th>Population</th>
<th>Prevalence</th>
<th>Density (No. per cm of tail)</th>
<th>Range (No. per cm of tail)</th>
<th>Prevalence</th>
<th>Density (No. per g fat)</th>
<th>Range (No. per g fat)</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inwood</td>
<td>100%</td>
<td>Mean: 91.6 (± 17.7)</td>
<td>13.7-325.9</td>
<td>100%</td>
<td>Mean: 1994.7 (± 379.8)</td>
<td>108.8-7031.4</td>
<td>81.0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median: 63.5 (44.4, 116.7)</td>
<td></td>
<td></td>
<td>Median: 1434.8 (497.4, 3349.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snake Island</td>
<td>100%</td>
<td>Mean: 48.1 (± 9.0)</td>
<td>2.3-160.9</td>
<td>100%</td>
<td>Mean: 1465.8 (± 216.1)</td>
<td>265.8-4561.0</td>
<td>78.3%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median: 38.5 (17.7, 55.6)</td>
<td></td>
<td></td>
<td>Median: 1303.0 (787.7, 1947.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fisher Exact test</td>
<td>P = 1.00</td>
<td>Rank Sum U = 122.0</td>
<td></td>
<td>Fisher Exact test</td>
<td>P = 1.00</td>
<td>Rank Sum U = 219.0</td>
<td>Fisher Exact test</td>
</tr>
</tbody>
</table>
**Table 3.3.** Prevalence, density, and range for parasites found in the lung of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) and plains garter snakes (*Thamnophis radix*) in Manitoba, Canada. Densities are shown as mean values (± SE) and median values (25th, 75th percentiles).

<table>
<thead>
<tr>
<th>Species</th>
<th>Rhabdias sp.</th>
<th></th>
<th></th>
<th>Lechriorchis sp.</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prevalence</td>
<td>Density (No. per g lung tissue)</td>
<td>Range</td>
<td>Prevalence</td>
<td>Density (No. per g lung tissue)</td>
<td>Range</td>
</tr>
<tr>
<td>Red-sided garter snake</td>
<td>98.3%</td>
<td>Mean: 34.4 (± 2.7)</td>
<td>0.987</td>
<td>71.7%</td>
<td>Mean: 23.9 (± 5.4)</td>
<td>0.197.0</td>
</tr>
<tr>
<td>(<em>T.s.parietalis</em>)</td>
<td></td>
<td>Median: 29.9 (20.5, 47.6)</td>
<td></td>
<td></td>
<td>Median: 8.1 (0.0, 22.9)</td>
<td></td>
</tr>
<tr>
<td>Plains garter snake</td>
<td>100%</td>
<td>Mean: 17.5 (± 5.3)</td>
<td>1.6-125.0</td>
<td>45.8%</td>
<td>Mean: 5.0 (± 1.8)</td>
<td>0.30.5</td>
</tr>
<tr>
<td>(<em>T.radix</em>)</td>
<td></td>
<td>Median: 9.9 (5.3, 18.5)</td>
<td></td>
<td></td>
<td>Median: 0.0 (0.0, 3.2)</td>
<td></td>
</tr>
<tr>
<td>Fisher Exact Test</td>
<td></td>
<td></td>
<td></td>
<td>X² = 3.921, 1 d.f.</td>
<td>Rank Sum</td>
<td>U = 287.0</td>
</tr>
<tr>
<td>P = 1.000</td>
<td></td>
<td></td>
<td></td>
<td>(T.s.p: N = 59, T.r.: N = 23)</td>
<td>(T.s.p: N = 60, T.r.: N = 24)</td>
<td></td>
</tr>
<tr>
<td>(T.s.p: N = 60, T.r.: N = 23)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rank Sum</td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.4. Prevalence, density, and range for parasites found in the tail, visceral fat, and gastrointestinal tract of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) and plains garter snakes (*Thamnophis radix*) in Manitoba, Canada. Densities are shown as mean values (± SE) and median values (25th, 75th percentiles).

<table>
<thead>
<tr>
<th>Species</th>
<th>Alaria spp.</th>
<th>Visceral Fat Metacercariae</th>
<th>Cestodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prevalence</td>
<td>Density (No. per cm of tail)</td>
<td>Range</td>
</tr>
<tr>
<td>Red-sided garter snake</td>
<td>100%</td>
<td>Mean: 74.2 (± 8.2)</td>
<td>0.9-271.0</td>
</tr>
<tr>
<td>(<em>T.s.parietalis</em>)</td>
<td></td>
<td>Median: 57.1 (19.1, 119.9)</td>
<td></td>
</tr>
<tr>
<td>Plains garter snake</td>
<td>100%</td>
<td>Mean: 160.7 (± 21.9)</td>
<td>21.3-409.9</td>
</tr>
<tr>
<td>(<em>T.radix</em>)</td>
<td></td>
<td>Median: 140.9 (79.9, 232.8)</td>
<td></td>
</tr>
<tr>
<td>Fisher Exact Test</td>
<td></td>
<td>Fishcer Exact Test</td>
<td>Rank Sum</td>
</tr>
<tr>
<td>P = 1.00</td>
<td></td>
<td>U = 331.0</td>
<td>U = 185.0</td>
</tr>
</tbody>
</table>
Table 3.5. Models of body condition, testes mass, and sperm counts for male red-sided garter snakes (*Thamnophis sirtalis parietalis*) from two populations in Manitoba, Canada. Finals models shown were selected via forward stepwise regression with AIC criteria. Body condition was calculated as the residuals from a regression of ln mass on ln SVL (snout-vent length). Testes mass and sperm counts are represented as the residuals from regressions of ln testes mass on ln SVL and square root transformed sperm counts on ln SVL, respectively. Visceral fat mass was natural log transformed and helminth densities were square root transformed.

<table>
<thead>
<tr>
<th>Population</th>
<th>Dependent Variable</th>
<th>Predictors in Final Model</th>
<th>Overall Model Fit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inwood</strong></td>
<td>Body Condition</td>
<td>Visceral fat mass (coefficient: 0.704, P &lt; 0.001) Rhabdias density (coefficient: -0.099, P = 0.067)</td>
<td>Adj. $R^2 = 0.805$, $F_{2,17} = 40.21$, P &lt; 0.001</td>
</tr>
<tr>
<td>(N = 20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testes Mass</td>
<td>Body condition</td>
<td>(coefficient: 0.744, P = 0.002)</td>
<td>Adj. $R^2 = 0.399$, $F_{1,17} = 12.97$, P = 0.002</td>
</tr>
<tr>
<td>(N = 19)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm</td>
<td>Lechriorchis density (coefficient: -0.122, P = 0.108)</td>
<td>Adj. $R^2 = 0.089$, $F_{1,18} = 2.86$, P = 0.108</td>
<td></td>
</tr>
<tr>
<td>(N = 20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Snake Island</strong></td>
<td>Body Condition (N = 22)</td>
<td>Visceral fat mass (coefficient: 1.088, P &lt; 0.001) Alaria density (coefficient: -0.107, P = 0.036)</td>
<td>Adj. $R^2 = 0.691$, $F_{2,19} = 24.44$, P &lt; 0.001</td>
</tr>
<tr>
<td>Testes Mass</td>
<td>Body condition</td>
<td>(coefficient: 0.801, P &lt; 0.001) Alaria density (coefficient: 0.126, P = 0.012) Rhabdias density (coefficient: 0.133, P = 0.046)</td>
<td>Adj. $R^2 = 0.692$, $F_{3,18} = 16.70$, P &lt; 0.001</td>
</tr>
<tr>
<td>(N = 22)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm</td>
<td>Alaria density</td>
<td>(coefficient: 0.253, P &lt; 0.001) Rhabdias density (coefficient: 0.204, P = 0.027) Lechriorchis density (coefficient: 0.086, P = 0.036)</td>
<td>Adj. $R^2 = 0.418$, $F_{3,18} = 6.03$, P = 0.005</td>
</tr>
<tr>
<td>(N = 22)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.6. Models of body condition, testes mass, and sperm counts for male red-sided garter snakes (*Thamnophis sirtalis parietalis*) and plains garter snakes (*Thamnophis radix*) in Manitoba, Canada. Finals models shown were selected via forward stepwise regression with AIC criteria. Body condition was calculated as the residuals from a regression of ln mass on ln SVL (snout-vent length). Testes mass and sperm counts are represented as the residuals from regressions of ln testes mass on ln SVL and square root transformed sperm counts on ln SVL, respectively. Visceral fat mass was natural log transformed and helminth densities were square root transformed.

<table>
<thead>
<tr>
<th>Species</th>
<th>Dependent Variable</th>
<th>Predictors in Final Model</th>
<th>Overall Model Fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red-sided garter snake</td>
<td>Body Condition</td>
<td>Intercept only</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Testes Mass</td>
<td>Intercept only</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sperm</td>
<td>VF metacercariae density (coefficient: -0.039, P = 0.060)</td>
<td>Adj. $R^2 = 0.288$, $F_{2,15} = 4.29$, P = 0.031</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Rhabdias</em> density, (coefficient: 0.391, P = 0.075)</td>
<td></td>
</tr>
<tr>
<td>Plains garter snake (T.radix)</td>
<td>Body Condition</td>
<td><em>Lechriorchis</em> density (coefficient: 0.169, P = 0.113)</td>
<td>Adj. $R^2 = 0.216$, $F_{2,20} = 4.023$, P = 0.034</td>
</tr>
<tr>
<td></td>
<td>Testes Mass</td>
<td><em>Alaria</em> density (coefficient: -0.157, P &lt; 0.001)</td>
<td>Adj. $R^2 = 0.470$, $F_{2,20} = 10.750$, P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Sperm</td>
<td><em>Rhabdias</em> density (coefficient: 0.265, P = 0.003)</td>
<td>Adj. $R^2 = 0.407$, $F_{2,20} = 8.562$, P = 0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Body condition (coefficient: 0.524, P = 0.005)</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 4: Changes in reproductive investment and hormone levels in response to an acute immune challenge

Emily J. Uhrig, Christopher R. Friesen, Leslie A. Blakemore, Deborah I. Lutterschmidt, Robert T. Mason

Abstract:
Tradeoffs often exist between the reproductive and immune systems because both are crucial to fitness and costly in terms of energy expenditure. Elevated immune activity, therefore, often depresses reproductive investment or, alternatively, increased reproductive investment comes at the expense of immunity. Such tradeoffs are often hormonally mediated and involve complex interactions among the reproductive, immune, and endocrine systems which have been most frequently investigated in taxa that are associated breeders. Here we present a study using a lipopolysaccharide (LPS, a non-pathogenic compound derived from bacteria) immune challenge to investigate reproductive-immune tradeoffs in a reptile with a dissociated breeding pattern where gametogenesis and peak hormone levels occur outside the breeding season. Our results indicate that male red-sided garter snakes (*Thamnophis sirtalis parietalis*) injected with LPS exhibit decreased reproductive investment demonstrated by reduced courtship and mating as well as reduced copulatory plug mass when mating does occur. Hormonal changes for LPS-treated males compared to controls included elevated plasma corticosterone and reduced androgens. Thus, hormonal mediation appears to underlie the observed depression of reproductive investment induced by the immune challenge.
4.1 Introduction

Conflict between the reproductive and immune systems is an important focus of evolutionary ecology as both systems are crucial to an individual’s fitness. The implications of this conflict, however, are not uniform across species. When faced with a potential pathogen, some species elevate immune function at the expense of reproduction (e.g. blue tits - Råberg et al. 2000; dragon lizards - Uller et al. 2006; cricket frogs - McCallum and Trauth 2007; flycatchers - Ilmonen et al., 2000; dung beetles - Reaney and Knell, 2010). Such depression of current reproductive investment (e.g. reductions in offspring number, gamete production, parental care) may help ensure survival for future reproduction (Stearns, 1992; Forbes 1993; Ilmonen et al 2000; Fedorka 2014). Alternatively, and perhaps more commonly noted in the literature, organisms may have low immune activity while increasing investment in reproduction (Nordling et al. 1998; Adamo et al. 2001; McKean and Nunney 2001; French et al. 2007). This strategy focuses resources on current reproductive investment (Forbes 1993; Sheldon and Verhulst 1996; Fedorka 2014). The ultimate goal of either strategy is to maximize lifetime reproductive fitness, and the strategy employed will likely depend on factors such as other life history traits of the organism in question (e.g. semelparity vs. iteroparity), sex and/or age of the organism, and the risk and type of infection they face. (Stearns 1992; Forbes 1993; Ricklefs and Wikelski 2002; Martin et al. 2008; Fedorka 2014).

The proximate mechanisms behind immune-reproduction tradeoffs are generally thought to be related to resource allocation (Sheldon and Verhulst 1996; Lochmiller and Deerrenberg 2000; Zera and Harshman 2001; Martin et al. 2008). That
is, both reproduction and immunity are energetically expensive processes drawing from the same finite pool of resources within an organism (Lochmiller and Deerenberg 2000; Zera and Harshman 2001; Martin et al. 2003; French et al. 2007; Martin et al. 2008). As such, they both cannot be simultaneously maximized because resources utilized for one will no longer be available for the other. However, resource independent mechanisms such as immunopathology or direct hormonal effects may also have a role in the conflict between immunity and reproduction (Råberg et al. 1998; Flatt et al 2005; French et al 2007; Harshman and Zera 2007).

Endocrine mediation of interactions between reproduction and the immune system have received considerable attention in the literature, with the interplay between sex steroid hormones and the immune system being particularly well studied (Ahmed et al. 1985; Klein 2000a; Klein 2000b; Martin et al. 2008). Testosterone, for example, that is elevated during the breeding season in many taxa, is generally cited as having immunosuppressive effects, which may help account for higher instances of disease and parasitism noted in males of many species (Folstad and Karter 1992; Poulin 1996; Klein 2000a; Alonso-Alvarez et al. 2009; Chow et al. 2015). Sex steroid hormones, however, are not the only endocrine mediators of immune-reproduction interactions. Glucocorticoids (e.g. corticosterone) also have major influences on the immune and reproductive systems. For instance, corticosterone, which is elevated during the breeding season for many species, is often associated with reduced immune function (Klein 2000a; Romero 2002; Klein 2004; French et al. 2007). However, the glucocorticoid-immune relationship is likely more complex than just immunosuppression. Rather, glucocorticoids appear to be important immune
modulators capable of enhancing, redistributing, and/or suppressing immune activity in order to fine tune the response to an infection (McEwen et al. 1997; Dhabhar 2002).

Although the reproductive consequences of immune challenges have been investigated in numerous studies, the vast majority have focused on taxa that display associated reproductive patterns. In associated breeders, sex hormone levels are highest, and gamete production occurs, during the breeding period. To the best of our knowledge, no studies have assessed reproductive-immune-endocrine interactions in species with a dissociated reproductive pattern where peak sex hormone levels and gamete production occur outside the breeding season (Crews 1984; Crews et al. 1984). The current study, therefore, seeks to broaden understanding of immune-reproduction conflict by investigating the consequences of an immune challenge in a reptile with a dissociated breeding pattern. In such a species, the temporal decoupling of elevated sex hormones and gametogenesis from mating activity allows us to examine the implications of an immune challenge within the breeding season but with naturally low testosterone, which could relax testosterone-mediated immunosuppression.

Red-sided garter snakes (Thamnophis sirtalis parietalis) range throughout the central United States and Canada and hibernate throughout the winter in underground hibernacula (Aleksiuk and Stewart 1971). With the warming of spring, the snakes emerge en masse and begin a short one month breeding season after which they migrate to summer feeding grounds before returning to hibernacula in the fall (Aleksiuk and Stewart 1971). During the breeding season, the operational sex ratio is
highly skewed towards males, which are the first to emerge from hibernation (Gregory 1974). As females emerge, each is quickly surrounded by numerous males forming a ‘mating ball’ which can consist of as many as 100 males around a single female (Gregory 1974). Male courtship is very vigorous and energetically expensive (Shine et al. 2001; Friesen et al., in press). Stereotypical courtship behavior involves males chin-rubbing along the female’s dorsum, aligning their bodies with the female, and caudocephalic waving (Crews et al. 1984; Moore et al. 2000). Male-male competition manifests in the form of ‘tail wrestling’ where males use their tails to maintain position near the female’s cloaca (Shine et al. 1999). During copulation, males deposit a gelatinous copulatory plug in the female’s cloaca (Devine 1975; Shine et al. 2000a; Friesen et al. 2013). The plug is crucial for male fitness as it not only acts as a mate guarding device (Shine, et al. 2000a), but also prevents sperm leakage and acts as a spermatophore containing the vast majority of sperm ejaculated during mating (Friesen et al. 2013). The plug dissolves over a 48 hour period in the female’s vaginal pouch, releasing the sperm which can then travel up the female’s oviducts (Friesen et al. 2013).

Testosterone levels of male garter snakes are elevated during the late summer and autumn (Krohmer et al. 1987; Lutterschmidt and Mason 2009). Spermatogenesis occurs during autumn as well with sperm then being stored over winter for use the subsequent spring (Krohmer et al. 1987). Thus, during the spring breeding season, a male’s sperm store is finite and he cannot replenish it until the following autumn. In contrast, the gelatinous matrix of the copulatory plug is derived from the renal sexual segment of the kidney (Friesen et al. 2013), which is hypertrophied in males during
the breeding season (Krohmer et al. 1987; Krohmer 2004a). This plug material can be replenished. However, the likelihood of producing a plug decreases significantly after more than two successive matings (Friesen et al. 2013) and the production of the plug material clearly has an energetic cost (Friesen et al. in press). As red-sided garter snakes are aphagous during the breeding season (O’Donnell et al. 2004), a male’s energy resources are limited to the fat reserves he has upon emergence from hibernation. Therefore, while males do not devote resources to spermatogenesis during the spring breeding season, plug production and vigorous courtship draw from the limited resources and males exhibit rapid mass loss during the breeding season (Shine and Mason 2005).

In contrast to the low levels of sex steroids in male garter snakes during the breeding season, glucocorticoid levels are elevated, likely facilitating the mobilization of energy from stored fat during their aphagous period (Moore et al. 2000; Cease et al. 2007). When experiencing additional stress (e.g., capture stress), male mating behavior is unabated in spite of further increases in plasma corticosterone (Moore et al. 2000). Such a decoupling of hormonal and behavioral stress responses is believed to be an adaptation for coping with adverse environmental conditions and a short breeding season (Moore et al. 2000). Exogenous doses of corticosterone, however, suppress male courtship behaviors indicating that there is a threshold for the behavioral stress response (Moore and Mason 2001; Lutterschmidt et al. 2004).

In the current study, we utilized lipopolysaccharide (LPS) injections to assess the effects of an acute immune challenge on reproduction in male garter snakes. LPS is a bacterial-derived endotoxin used to elicit an immune response, specifically the
acute phase response (Suffredini et al. 1999), without being pathogenic itself. Its non-pathogenic nature has led to LPS being used in numerous studies across a variety of vertebrate taxa to investigate costs of immune activation (lizards - Uller et al. 2006; López et al. 2009; toads - Llewellyn et al. 2012; birds - Bonneaud et al. 2003; Romano et al. 2011; rodents - Prendergast 2003; bats - Schneeberger et al. 2013; primates - Canale and Henry 2011). In our study, we investigated whether immune-challenged males alter 1) intensity of courtship behaviors and/or 2) characteristics such as sperm counts or copulatory plug mass. Additionally, we determined plasma levels of corticosterone and androgens to evaluate whether changes in reproductive investment might be mediated by hormonal changes in response to the immune challenge.

4.2 Methods

4.2.1. Study Population

All red-sided garter snakes used in this study were indiscriminately collected by hand during the boreal spring breeding season. Collections were made at a den site (Inwood) located in the Interlake region of Manitoba, Canada (50°31’58”N; 97°29’71”W). Animals were transported from the den approximately 16 kilometers to the Chatfield Research Station where all behavioral trials were conducted. Animals were housed outdoors in seminatural arenas [1m x 1m x 1m nylon construction as in (Moore and Mason 2001)] with males and females kept separately until behavioral trials. Water was provided *ad libitum*, but food was not as the snakes are aphagous during the breeding season (O’Donnell et al. 2004).
4.2.2. Courtship and Mating

On day 1 of the experiment (the same day males were collected), courting male snakes were placed into seminatural arenas. Unmated females (N = 2) were introduced to each arena and the snakes were allowed to interact and mate naturally. When a mating occurred, a timer was started to measure copulation duration; after one minute in copulo, the mating pair was carefully moved to a small circular arena (45 cm dia. x 75 cm height) where they could be more easily observed (e.g. Friesen et al. 2014). The timer was stopped when copulation ceased. After removing a mating pair, a new unmated female was added to the arena. A total of 54 matings were obtained in this manner, all on the same day.

At the end of day 1, the mated males were randomly assigned to either a treatment or control group (N = 27 per group). The treatment group received an intraperitoneal injection of lipopolysaccharide (LPS from E. coli 0127:B8; Sigma-Aldrich). LPS was dissolved in phosphate buffered saline (0.4 mg/ml) and administered at a dose of 2.52 μg per gram body weight. Males in the control group were injected intraperitoneally with an equivalent volume of phosphate buffered saline. The LPS dose we used was similar to that used in other studies of immune challenges in reptiles such as Uller et al. (2006) who found the LPS dose to result in decreased egg mass for female dragon lizards (Ctenophorus fordi). In our study, all males were injected within a one hour period, which was between 2 and 6 hours after mating. Our reasons for using only mated males in the experiment were two-fold. First, it ensured that all of the males were actively courting to the degree that they would mate. Second, LPS would be unlikely to affect plug mass in a first mating
because males would most likely have already invested energy in producing the plug material prior to being injected. Rather it was more plausible that an effect of LPS would depress the ability of mated males to replenish plug material.

On day 2, beginning approximately 17 hours post-injection, males were given the opportunity to mate a second time. Treatment and control males were randomly assigned to one of three seminatural arenas such that each arena contained 9 treatment and 9 control males. Each male was marked by affixing a small, numbered piece of adhesive tape (Nexcare®) to his head to facilitate identification from a distance without disturbing the animals. Beginning at 11:30am, two females were introduced into each arena. One observer, blind to the treatments, was assigned to each arena. Courtship intensity was scored for each male during three minute focal samples beginning at 11:45am and continuing every 30 minutes thereafter for a total of ten observation periods. Courtship scores were based on the previously established ethogram of male garter snake courtship behavior where scores range from 0 (no reproductive behavior) to 5 (copulation) (Moore et al. 2001; Table 4.1). Courtship intensity was measured as a male’s highest courtship score achieved during the day’s observations (Shine et al. 2012). As matings occurred, a timer was started and after one minute the mating pair was moved to a small circular arena to measure copulation duration as previously described. Following the removal of a mating pair from an arena, a new female and non-experimental male were added to maintain the same sex ratio. The added males had their cloacae taped to prevent mating.
4.2.3. Blood Collection and Hormone Analysis

Following the conclusion of the final behavioral observation period on day 2, approximately 23 hours post-injection, a blood sample (100 μl) was taken from the caudal vein of each male (N = 54) within 2 min using a heparinized 1 cm³ syringe with a 25-gauge needle. Blood samples were kept cool (~1 °C) until being centrifuged to separate the plasma which was transferred into a new microcentrifuge tube and frozen at -4 °C. Samples were placed on dry ice for transport to Portland State University and stored at -80 °C until further analysis. Direct radioimmunoassay for corticosterone and androgens was carried out using previously established protocols described in detail by (Lutterschmidt et al. 2004; Lutterschmidt and Mason 2008; Lutterschmidt and Mason 2009). As our testosterone antibody exhibits significant crossreaction with 5-α-dihydrotestosterone (63% crossreactivity; Fitzgerald Industries International, Acton, MA, USA), our direct assay measures both plasma testosterone and 5-α-dihydrotestosterone concentrations. Therefore, the data we present here reflect total androgen concentrations as in Lutterschmidt and Mason (2005, 2009). To determine individual sample recovery, each plasma sample was spiked with 2,000 cpm of tritiated steroid and incubated 16-24 hrs; concentrations of both corticosterone and androgen were determined from the same plasma aliquot. Steroids were extracted from each plasma sample with anhydrous ethyl ether. The ether phase was removed and dried under nitrogen gas in a bead bath at 37°C. Hormone extracts then were reconstituted in 600 μl of phosphate-buffered saline. A 50 μl aliquot of each extracted and reconstituted sample was used to determine individual sample recovery. The remainder of each sample was allocated to two duplicate culture tubes per steroid
hormone being assayed. Serial dilutions of the standard curve (performed in triplicate), 0% bound (nonspecific binding), 100% bound, and all samples were incubated with 12,000 cpm 1,2,6,7-[3 H]corticosterone or 1,2,6,7-[3 H] testosterone (Perkin Elmer, Piscataway, NJ). Except for nonspecific binding samples, all tubes were also incubated with 100 μl antiserum at 4 °C for 18–24 h (corticosterone antibody 07120016 from MP Biomedicals, LLC, Solon, OH; testosterone antibody 20R-TR018w from Fitzgerald Industries International, Acton, MA). Dextran-coated charcoal was used to separate unbound steroid from bound hormone. The bound steroid was decanted into scintillation vials and the radioactivity of each sample was quantified in a Beckman 6500 liquid scintillation counter. All hormone concentrations were corrected for individual recovery variation. Mean extraction efficiency was 89.0%. All samples were randomly distributed across assays. The mean intra-assay coefficient of variation was 14.3% for corticosterone and 13.0% for androgens. The inter-assay coefficient of variation was 15.5% for corticosterone and 18.1% for androgens. Mean limits of detectability were 31.4 pg/ml and 16.4 pg/ml for corticosterone and androgens, respectively. All plasma corticosterone concentrations were above the limits of detectability. In some cases (4 of 54 samples), androgen concentrations were below the limits of detectability; in these instances, the samples were assigned the limit of detectability (e.g. 16.4 pg/ml) in order to retain these samples in our statistical analyses.
4.2.4. **Copulatory Plug Collection and Sperm Counts**

For all matings that resulted in the deposition of a copulatory plug, the plug was removed within 5 minutes of the cessation of copulation by inserting a blunt probe gently into the female’s cloaca and moving it around the perimeter to loosen the plug (Friesen et al. 2013). The plugs were placed in pre-massed 1.5ml microcentrifuge tubes and kept on ice until the end of the day’s behavioral trials. Plug mass (± 0.001 g) was determined by weighing (A&D Fx-300i balance) the microcentrifuge tube containing the plug and then subtracting the mass of the vial. After weighing, 1ml of sperm washing medium (Irvine Scientific) was added to each vial and the plugs were refrigerated (4 °C) for 3 days to allow for plug dissolution and the liberation of the sperm (Friesen et al. 2013). Once the plugs were sufficiently dissolved, as evidenced by visible breakdown of the plug and a cloud of released sperm (Friesen et al. 2013; Friesen et al. 2014), each vial was shaken gently to homogenize the sample. A 2μl aliquot of the sample was placed on a Petroff-Hauser counting chamber at 10x magnification under an Olympus CX31 phase contrast compound microscope equipped with an Olympus DP-21 digital camera operated with CellSens software (Olympus). Overly concentrated samples (> approximately 100 sperm on the counting grid) were diluted with PBS. Once an appropriate concentration was achieved, a photo of the sperm on the counting grid was taken and saved in JPEG format; this was repeated for an additional two aliquots of the sample for a total of three replicates per sample. The photographed sperm were later counted at Oregon State University.
4.2.5. Statistics

As an estimate of body condition, we used residual scores from a linear regression of ln mass on ln snout-vent length (SVL; Shine and Mason 2005). This regression was based on data from 254 male snakes all collected in the same season (the 54 males from the current experiment plus an additional 200 males randomly collected for a separate study). Male mass, SVL, tail length, body condition, courtship intensity, sperm counts, and hormone data were analyzed using Student’s t-tests or, where data were not normally distributed, Mann-Whitney rank sum tests to make comparisons between treatment groups. Since previous studies have shown plug mass is correlated with male size (Shine et al. 2000a), we compared plug masses between treatments using ANCOVA with male mass as a covariate. Chi-square tests were used to compare the proportions of males remating as well as the proportion of males that did not display courtship behaviors. Given the ordinal nature of courtship scores, Spearman rank correlation was used to assess the relationship between courtship intensity and body condition as well as courtship intensity and hormones. Linear regression was used to assess correlations between body condition and copulation duration, plug mass, sperm counts, and hormones. The correlation between corticosterone and androgens as well as correlations between each of these hormones and copulation duration, plug mass, and sperm counts were also assessed via linear regression. All analyses were conducted in either SigmaPlot (version 12.5, Systat Software, Inc.) or XLStat (version 2013.4.8, Addinsoft).
4.3 Results

4.3.1. Courtship and Mating

The natural matings that occurred on day 1 resulted in a total of 54 mated males all of which deposited a copulatory plug during mating. The mated males were randomly assigned to either the LPS treatment or the control group. Neither male mass (Mann-Whitney rank sum, U = 309.0, P = 0.341), snout-vent length (SVL; Student’s t-test, t = 0.273, P = 0.786), tail length (Mann-Whitney rank sum, U = 351.0, P = 0.822), nor body condition (Student’s t-test, t = 1.654, P = 0.104) differed between treatment groups. Male mass, SVL, tail length, and body condition also did not differ between LPS-treated and control individuals within the arenas to which males were assigned for day 2 mating trials (P > 0.05 in all cases).

On day two of the experiment, 21 of the 54 males mated again (N = 4 LPS-injected males, N = 17 control males). The proportion of LPS-injected males that remated (i.e. achieved a courtship score of 5) was significantly less than the proportion of control males that did so ($\chi^2 = 11.221$, d.f. = 1, $P < 0.001$; Figure 4.1b), with less than 15% of LPS treated males mating again compared to nearly 63% of control males. Body condition did not differ between males that remated and those that did not (Student’s t-test, $t_{52} = 0.249$, $P = 0.804$). Copulation duration did not differ between the treatments (Student’s t-test, $t_{19} = 1.653$, $P = 0.118$) and there was also no difference in body condition between the LPS-injected and control males that remated (Student’s t-test, $t_{19} = 1.745$, $P = 0.097$). However, when all males were pooled together, higher body condition was correlated with longer copulation durations during the second mating (Linear regression, adj. $R^2 = 0.201$, $P = 0.024$).
Analyses including only the males that did not remate (i.e. courtship scores ≤ 4) indicated courtship intensity was significantly lower for the LPS-injected males compared to the controls (Mann-Whitney rank sum, $U = 62.0$, $P = 0.030$). Over 40% of LPS-injected males (11 individuals) did not exhibit courtship behaviors during any of the observation periods (i.e. scored zeros on the ethogram) whereas only 7.4% of control males (2 individuals) did not show courtship ($X^2 = 6.484$, d.f. = 1, $P = 0.011$; Figure 4.1a). Courtship intensity was not correlated with body condition (Spearman rank correlation, $\rho = -0.083$, $P = 0.645$).

4.3.2. Copulatory Plugs, Sperm Counts

Copulatory plugs were produced by 17 of the 21 males that mated on day 2; the four males that did not produce plugs were all control males. Considering only the males that did produce plugs on their second mating, plug mass was significantly lower for males that received LPS (ANCOVA with male mass as covariate, $P = 0.024$, Figure 4.2). These males did not differ from the control males in terms of plug mass from their pre-injection first mating (ANCOVA with male mass as covariate, $P = 0.452$). Sperm counts, however, did not differ significantly between LPS-injected and control males (Student’s t-test, $t_{15} = 0.444$, $P = 0.664$). Plug mass was not correlated with body condition in the second matings (Linear regression, adj. $R^2 = 0.052$, $P = 0.190$) or with sperm counts (Linear regression, adj. $R^2 = -0.067$, $P = 0.988$).
4.3.3. Hormone Analyses

Plasma levels of both testosterone and corticosterone varied significantly between LPS-treated and control males, but in opposite manners. With a median value of 128.63 ng/ml, males treated with LPS had plasma corticosterone levels that were approximately 1.8 times higher than control males for which median corticosterone was 70.55 ng/ml (Mann-Whitney Rank Sum, U = 59.00, P < 0.001, Figure 4.3a). Androgens, in contrast, were more than three times higher in control males compared to LPS males (0.15 ng/ml for LPS males vs. 0.50 for control males; Mann-Whitney Rank Sum, U = 95.50, P <0.001, Figure 4.3b). Regardless of treatment, males that remated on day 2 had lower corticosterone (Student’s t-test, t = 2.056, P = 0.045) and higher androgen levels (Mann-Whitney Rank Sum, U = 202.00, P = 0.011) compared to males that did not remate. Considering only males that did not remate, neither androgen nor corticosterone levels were correlated with courtship intensity (Spearman rank correlation: ρ = 0.069, P = 0.696 for androgen; ρ = -0.249, P = 0.154 for corticosterone). After levels of both hormones were natural-log transformed to help correct for non-normality, a linear regression of plasma levels of androgens and corticosterone indicated the negative correlation between the two hormones was significant (adj. R² = 0.113, P = 0.008). Further, higher corticosterone levels were correlated with shorter copulation durations (Linear regression, adj. R² = 0.185, P = 0.029), lower body condition (Linear regression, adj. R² = 0.117, P = 0.007), and lower plug mass (Linear regression, adj. R² = 0.374, P = 0.005). Corticosterone was not correlated with sperm count (Linear regression, adj. R² = -0.067, P = 0.977). Androgen level showed no significant correlation with copulation
duration, body condition, plug mass, or sperm count (Linear regressions, P >0.05 for all).

4.4 Discussion

When faced with an immune challenge during the breeding season, male garter snakes substantially altered reproductive behavior. Males receiving lipopolysaccharide (LPS) injections displayed both reduced courtship intensity and were less likely to remate compared to control males. These behavioral alterations associated with LPS treatment were accompanied by decreased copulatory plug mass, but sperm counts did not differ between treatments. Hormonal changes included increased corticosterone and decreased testosterone levels in LPS-treated males.

4.4.1. Effects of an Immune Challenge on Reproductive Investment:

The reduction in mating and courtship intensity we observed may reflect reallocation of resources to the immune system and away from reproductive behaviors (Lochmiller and Deerenberg 2000; Zera and Harshman 2001; Martin et al. 2008). Indeed, it has been suggested that the acute phase response (APR), which is induced by LPS injection, is the most energetically costly type of immune response (Klasing 2004; Owen-Ashley and Wingfield 2007). The vigorous courtship characteristic of male garter snakes is also energetically expensive and crucial to male fitness as males exhibiting more vigorous courtship have greater mating success (Shine et al. 2001; Shine et al. 2004a; Shine and Mason 2005). Therefore, the low proportion of LPS-treated males remating is likely a direct consequence of their
reduced courtship intensity. Previous studies have noted that male body condition is positively correlated with courtship intensity and/or mating success (Shine et al. 2000b; Shine et al. 2004a). However, in our study, body condition did not differ between treatment groups or between males that remated and those that did not, suggesting that the observed differences in reproductive behavior were not due to variation in body condition, but were a direct result of the immune challenge.

The decrease in copulatory plug mass of LPS-treated males also indicate a tradeoff between reproduction and the immune response. Copulatory plug material is produced by the renal sexual segment (RSS) of the kidney (Krohmer et al. 1987). During the spring breeding season, the RSS is hypertrophied in males and actively producing plug material that is stored in secretory vesicles in apocrine cells of the RSS (Krohmer 2004b; Krohmer 2004a; Aldridge et al. 2011); depleted stores of plug material are replenished after mating (Bishop 1959; Krohmer 2004a; Krohmer et al. 2004). This replenishment appears to be energetically costly because metabolic rates and lactate levels are higher for post-mating males compared to males that only engage in courtship and not plug production (Shine et al. 2004b; Friesen et al. in press). Therefore, lower plug mass in LPS-injected males is likely another reflection of resources being redistributed away from reproduction in the face of an immune challenge. As the plug prevents sperm leakage and functions as a mate guarding device (Shine et al. 2000a; Friesen et al. 2013), decreased plug mass could adversely affect male reproductive fitness.

In contrast to our results for plug mass, sperm counts did not differ between LPS-treated males and controls, although the small sample size for sperm counts from
LPS-treated males limited our ability to detect differences. However, that sperm numbers were unaffected by LPS treatment is not surprising given that spermatogenesis is completed by the autumn prior to hibernation (Crews 1984; Krohmer et al. 1987). Thus, resources had already been expended for producing sperm, and activating the immune system in spring did not influence sperm counts.

### 4.4.2. Hormonal Mechanisms of Immune Challenge:

The LPS-injected males in our study exhibited dramatically elevated plasma corticosterone compared to control males. Interestingly, male garter snakes are able to maintain courtship during times of stress and elevated corticosterone although there is a threshold corticosterone level above which mating behavior is depressed (Moore et al. 2000; Moore and Mason 2001; Lutterschmidt et al. 2004). Although comparing plasma hormone levels among different studies and years can be problematic, Moore and Mason (2001) showed treatment of male red-sided garter snakes with 50 μg of exogenous corticosterone resulted in mean plasma corticosterone levels of approximately 90 ng/ml and, further, treatment with such a dose of corticosterone greatly reduces courtship (Moore and Mason 2001). Our LPS treatment led to mean corticosterone levels well above 90 ng/ml while control males had corticosterone levels well below that concentration. Thus, in our study, LPS treatment likely resulted in corticosterone exceeding the threshold for a behavioral response and contributed to the inhibition of mating behaviors.

In contrast to elevated corticosterone, LPS treatment in our study was associated with a decrease in plasma androgens. Although the relationship between
corticosterone and androgens is somewhat equivocal for male red-sided garter snakes (Moore and Mason 2001; Lutterschmidt and Mason 2005), the negative relationship between corticosterone and androgens that we observed is in accordance with the findings of Moore et al. (2000). Based on Moore and Mason (2001) and Lutterschmidt et al. (2004) elevated corticosterone does not directly drive a decrease in androgens. Thus, the lower androgen levels of LPS-treated snakes may result from immune activation suppressing testosterone (O’Bryan et al. 2000; Boonekamp et al. 2008). The fitness relevance of immune-mediated androgen suppression is not immediately clear given that androgens do not directly alter mating behavior during the spring (Crews et al. 1984). However, as the RSS is influenced by androgens (Krohmer et al. 1987), the lower androgen levels of LPS-treated males may have contributed to the reduced masses of copulatory plugs produced by these males. Additionally, as testosterone tends to be immunosuppressive (Folstad and Karter 1992; Klein 2000a; Klein 2000b; Alonso-Alvarez et al. 2009), it may be that the low testosterone levels characteristic of the red-sided garter snakes’ dissociated breeding pattern permitted the occurrence of an immune response during the breeding season.

Like Moore et al. (2001), we found a negative correlation between corticosterone and body condition overall. However, the corticosterone response to LPS occurs independently of body condition, which did not vary between treatments. Indeed, increased glucocorticoid levels is typical of the acute phase response (Gabay and Kushner 1999; Owen-Ashley and Wingfield 2007), and have been shown to occur in response to LPS treatment for a number of species including rats (Rattus norvegicus; (Johnson et al. 1996), chickens (Gallus gallus; Shini et al. 2008), and
white-crowned sparrows (*Zonotrichia leucophrys*; Owen-Ashley et al. 2006). In the initial acute phase response, glucocorticoids stimulate immune activity while later on the hormones provide negative feedback for shutting down the immune response (McEwen et al. 1997; Dhabhar 2002; Owen-Ashley and Wingfield 2007). The immunosuppressive action of glucocorticoids, particularly their regulation of cytokines, is likely beneficial for avoiding immunopathology, whereby excessive or prolonged immune activity can negatively affect fitness (Fantuzzi and Ghezzi 1993; Johnson et al. 1996; Råberg et al. 1998; Graham et al. 2005). Thus, the observed corticosterone increase in LPS-treated snakes may, at least in part, represent a physiological braking mechanism on the immune response. Nonetheless, any benefits derived from activating the immune system and elevating corticosterone clearly come with the cost of reduced reproductive effort. Such a strategy seems to indicate sacrifice of current reproduction in favor of survival for future reproduction (Stearns 1992; Forbes 1993). However, as all males in our experiment had already mated at least once, it may be that constraints on immune investment due to reproduction were more relaxed due to their mating history. Indeed, for some insect taxa, mated individuals have increased resistance to pathogens compared to virgins (crickets, *Gryllus texensis* - Shoemaker et al. 2006; mealworm beetles, *Tenebrio molitor* - Valtonen et al. 2010), but vertebrate examples of this phenomenon are lacking. Thus, future studies using seasonal virgin male garter snakes could be informative.
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4.5 References


Table 4.1. Ethogram of male garter snake reproductive behavior (modified from Moore et al. 2001). Behaviors associated with scores of 2 or greater are only exhibited in a reproductive context.

<table>
<thead>
<tr>
<th>Courtship Score</th>
<th>Description of Male Behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No reproductive behaviors exhibited</td>
</tr>
<tr>
<td>1</td>
<td>Male investigates female</td>
</tr>
<tr>
<td>2</td>
<td>Male chin-rubs female, tongue flicks rapid</td>
</tr>
<tr>
<td>3</td>
<td>Male’s body is aligned with female</td>
</tr>
<tr>
<td>4</td>
<td>Male actively tail searches female, attempts cloacal apposition; possible caudocephalic waves.</td>
</tr>
<tr>
<td>5</td>
<td>Copulation occurs</td>
</tr>
</tbody>
</table>
Figure 4.1. The proportions of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) injected with either lipopolysaccharide (LPS) or saline (Control) that A) exhibited no courtship behaviors during any observation periods on day 2 of the experiment and B) mated on day 2 of the experiment. Asterisks denote statistically significant differences.
Figure 4.2. Copulatory plug masses for the second mating of male red-sided garter snakes injected with either lipopolysaccharide (LPS) or saline (control). After accounting for male size, plug masses were significantly different between treatments (ANCOVA, P = 0.024).
Figure 4.3. Plasma concentrations of A) corticosterone and B) androgens for male red-sided garter snakes injected with lipopolysaccharide (LPS) or saline (Control). Androgen concentrations are shown on a log scale to improve legibility of the graph. Asterisks denote statistically significant differences.
Chapter 5: Implications of repeated immune challenges in a capital breeder with prolonged hibernation

Emily J. Uhrig, Leslie A. Blakemore, Christopher R. Friesen, Robert T. Mason

Abstract:

Costly processes such as reproduction and immune activity generally tradeoff with one another. As both survival and reproduction are paramount to fitness, the nature of immune-reproduction tradeoffs can vary depending on what will maximize lifetime fitness. For capital breeders relying on stored resources for offspring production, immune challenges that impede resource accumulation can hinder future reproduction. Additionally, if there is an intervening hibernation period between resource acquisition and breeding, then the animal must accrue enough resources to survive hibernation as well as reproduce. In the current study, we used lipopolysaccharide (LPS, a non-pathogenic compound derived from bacteria) immune challenges to evaluate reproductive-immune tradeoffs in a capital breeder with prolonged hibernation: the red-sided garter snake (Thamnophis sirtalis parietalis). Our results did not clearly demonstrate a reproductive-immune tradeoff. Males did not exhibit reduced testes mass or sperm counts when treated with LPS. Females treated with LPS were not less likely to become gravid compared to controls. Although a lower proportion of offspring from LPS-treated females survived to 60 days post-birth, LPS-treated females had more offspring per litter than control females and offspring size did not differ between treatments. The absence of clear tradeoffs may be due to the availability of food resources during the experiment. In
the LPS treatment, increased food consumption was exhibited by both gravid females and the males from which testes and sperm were obtained. These results indicate that snakes in the midst of reproductive activity (i.e., gravid females, males undergoing spermatogenesis) compensated from immune activation by increasing food consumption to maintain current reproductive processes. However, anorexia during infections tends to have survival benefits and, indeed, we found that non-gravid females treated with LPS showed reduced food intake which may lead to improved survival for future reproduction. Interestingly, the increased food consumption of males did not translate into greater fat stores, but rather higher liver masses which may be indicative of immunopathological changes which should be explored in future studies.

5.1 Introduction

Activating immune responses is an energetically expensive endeavor (Beisel 1977; Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Sandland and Minchella 2003; Martin et al. 2003; Martin et al. 2008; Demas et al. 2012). When a pathogen is encountered, immune activity often becomes elevated while other processes are depressed in an effort to ensure survival (Ilmonen et al. 2000; Råberg et al. 2000; Soler et al. 2003; Uller et al. 2006). However, reproduction is also key to fitness and, consequently, immune responses may be dampened during reproductive periods likely due to increased resources expended on the production of offspring (Nordling et al. 1998; Adamo et al. 2001; McKean and Nunney 2001; French et al.
Therefore, tradeoffs often exist between immune activity and other costly processes such as growth and reproduction (Stearns 1992; Lochmiller and Deerenberg 2000; Zera and Harshman 2001; French et al. 2007a; Martin et al. 2008). The degree of immune activity in response to an invading pathogen depends on the best strategy for maximizing lifetime fitness. The best strategy, in turn, depends on a variety of other factors relating to attributes of the host (e.g., sex, age), the pathogen (e.g., virulence, length of infection), and the environment (e.g., season, resource availability) (Stearns 1992; Forbes 1993; Ricklefs and Wikelski 2002; Sandland and Minchella 2003; Martin et al. 2008).

Allocation of resources is arguably the most commonly cited mediator of tradeoffs with the immune system (e.g., Sheldon and Verhulst 1996; Norris and Evans 2000; French et al. 2009). That is, immune activity and other expensive processes such as reproduction cannot be simultaneously maximized because there are generally not sufficient energetic resources to do so (Ahmed et al. 2002; Klasing 2004; French et al. 2009). In support of this view are studies where immune-reproduction tradeoffs are observed under conditions of limited food resources, but not when ample resources are available (e.g., French et al. 2007b; Simmons 2012). French et al. (2007a) refer to the resource dependent relationship as “facultative regulation” of the tradeoff as opposed to “obligate regulation” where a tradeoff is observed regardless of resource availability (e.g., sex hormone induced immunosuppression – Schuurs and Verheul 1990; Flatt et al. 2005). The implication of facultative regulation is that immune challenged individuals will increase food
intake to compensate for the resources expended on immune activity. However, anorexia is a common sickness behavior and reduced food intake, although somewhat counterintuitive, has been shown to improve survival of infected individuals (Wing and Young 1980; Exton 1997). Some components of the immune system (e.g. macrophage and natural killer cell activity) are actually enhanced by fasting (Wing et al. 1983; Exton 1997). Thus, the benefits of anorexia may mediate immune-reproduction tradeoffs. However, few studies, particularly of wild taxa, actually monitor food intake as well as evaluate reproductive investment during immune challenges.

Immune challenges need not occur during the actual mating season to depress reproduction and this is especially true for capital breeders and species with dissociated reproductive patterns. For dissociated breeders, peak gonadal activity is temporally dissociated from the mating season (Crews et al. 1984; Lutterschmidt 2012). Therefore, infections occurring outside the breeding season but during periods of gonadal activity could depress reproductive investment in the subsequent breeding season via, for example, alterations of gametogenesis.

For capital breeders, production of offspring mainly depletes stored resources acquired prior to breeding (Stearns 1992; Shine and Schwarzkopf 1992; Jönsson 1997; Bonnet et al. 1998), but immune challenges can still influence how these resources are allocated to current reproduction (e.g., Hanssen 2006). Additionally, if immune activation leads to altered behaviors and/or food intake (e.g., infection induced anorexia (Exton 1997)), then infected individuals may accumulate fewer
resources and be poorly prepared for future reproduction (i.e., have less ‘capital’ to invest in the subsequent breeding season). Accumulating sufficient resources may be even more critical if there is an intervening hibernation period between feeding and reproduction as is the case with capital breeders living in colder climates. Indeed, studies of frogs along latitudinal gradients have noted greater energy reserves for species occurring at colder northern latitudes [European common frog, *Rana temporaria* (Jönsson et al. 2009); Chinese brown frog, *Rana chensinensis* (Chen et al. 2011)]. Immune activation during the feeding period of species undergoing prolonged hibernation, therefore, could adversely affect survival during hibernation if resource accumulation was impeded. However, infections not dealt with prior to hibernation could affect overwinter survivability as immune function is often suppressed during hibernation, allowing infections to be particularly pathogenic (Bouma et al. 2010; Hueffer et al. 2011).

As a capital breeder living in northern latitudes, the red-sided garter snake (*Thamnophis sirtalis parietalis*) makes an excellent model for investigating the reproductive consequences of immune challenges in a species with prolonged hibernation. In the more northern margins of their range, red-sided garter snakes hibernate for over seven months of the year in underground hibernacula (Gregory 1977). In the boreal spring, the snakes emerge *en masse* for a short breeding season lasting approximately four weeks followed by migration to summer feeding grounds and a return to hibernation in the autumn (Gregory and Stewart 1975; Gregory 1977). During hibernation and the subsequent mating season, snakes are aphagous
(O’Donnell et al. 2004), and, as capital breeders, they must rely on energy stores acquired during the previous summer’s feeding season to sustain them throughout these periods (Gregory 2006). Therefore, factors affecting the snakes’ ability to maximize food intake during the summer period could influence their overwinter survival and ensuing reproductive capabilities. While the summer feeding overlaps with pregnancy, this ‘income’ does not appear to greatly influence offspring production (Gregory 2006). However, food intake while gravid may help support the elevated metabolic costs of pregnancy (Birchard et al. 1984; Lourdais et al. 2002; Gregory 2006; but see Ladyman et al. 2003). As a dissociated breeder, summer and early fall represent periods of important reproductive events for red-sided garter snakes. In early summer, females undergo vitellogenesis and ovulation approximately six weeks post-emergence and give birth at the summer feeding grounds (Gregory 1977; Whittier et al. 1987). For male garter snakes, peak testicular activity and spermatogenesis occurs during late summer through early autumn (Krohmer et al. 1987). Additionally, although the majority of mating occurs in spring, autumn matings also occur (Aleksiuk and Gregory 1974; Mendonça and Crews 1989; Friesen et al. 2014)). Therefore, immune challenges occurring during the summer and autumn seasons could still affect the snakes’ reproduction.

In the current study, we used a series of lipopolysaccharide (LPS) injections as a non-pathogenic repeated immune challenge for both male and female red-sided garter snakes. LPS is an endotoxin compound derived from the cell wall of gram-negative bacteria and has been used in a wide variety of taxa including several species
5.2 Methods

5.2.1. Animal Collection and Housing

All red-sided garter snakes used in this study (N = 48 males, 50 females) were collected during the spring breeding season at a hibernaculum of over 35,000 snakes (Shine et al. 2006) in the Interlake region of Manitoba, Canada (50°31’58”N; 97°29’71”W). All females used in this study had mated naturally in the field as evidenced by the presence of a copulatory plug occluding the cloaca (Shine et al. 2000). Snakes were transported to Oregon State University where they were housed in 10-gallon aquaria in a light and temperature controlled environmental chamber (13:11 hr, L:D; 24:18°C). A 40W incandescent lamp illuminated for 5 hrs/day was suspended over half of each tank enabling the snakes to bask and naturally
thermoregulate. Males and females were housed separately at all times with no more than 6 males or 3 females per tank.

5.2.2. Feeding and Appetite

Snakes were fed twice weekly, earthworms or (thawed from frozen) salmon fry on alternating weeks, and provided water ad libitum. On feeding days, dishes containing approximately 40g of food were placed in each tank. If the initial food portion was completely consumed, the dish was refilled; this process was repeated until the snakes no longer showed interest in the food (e.g. they moved away from the food and coiled themselves under the basking lamp). To assess the effects of LPS treatment on food intake, all snakes were weighed weekly (Ohaus balance) before and after feeding over the course of 10 weeks. Water dishes were removed from the aquaria during the feeding period so that mass changes associated with food intake were not confounded by water intake.

5.2.3. LPS Injections

Males and females were randomly allocated to either a treatment or control group. Animals in the treatment group received an intraperitoneal injection of lipopolysaccharide (LPS; from E. coli 0127:B8 (Sigma-Aldrich)). LPS was dissolved in phosphate buffered saline (0.4 mg/ml) and administered at a dose of 2.52 μg per gram body weight; controls received an equivalent volume of phosphate buffered saline injected intraperitoneally. This LPS dose was similar to that used by other
studies of immune challenges in reptiles (e.g. Uller et al. 2006). Injections were administered every three weeks beginning 10 and 11 June, for males and females respectively, and continuing throughout the summer with females receiving their final injection on 19 August. For males, injections continued into the autumn with the last injection administered on 13 November; the injection period was longer for males because spermatogenesis continues into the autumn.

5.2.4. Gravid Females

Females were allocated randomly to aquaria so that tanks contained both control and treated individuals (2-3 females per aquarium; 17 aquaria total). Although all females had mated, it is typical of this population that not all mated females become gravid and give birth within the same mating season (Gregory 2006; Friesen et al 2014). By mid-July we were able to assess gravidity via palpation of the females’ ventral surfaces. For gravid females the presence of vitellogenic follicles could be felt as a series of pronounced nodules along the ventrum (Whittier and Crews 1986; Gregory 2006). Housing arrangements were modified at this time such that only one gravid female was present in each aquarium, thus we could be sure of the maternal identity upon finding a litter. Aquaria were checked daily for offspring. Following their birth, all offspring from a litter were removed from the mother’s aquarium and placed in a separate smaller aquarium. In addition to fully formed offspring, some females passed undeveloped yolk sacs. These yolk sacs were also collected and weighed. All offspring including stillborns were weighed, measured, and sexed. Litters were maintained in separate aquaria and checked daily for
mortality over the course of the next two months. One LPS-treated female developed an obstruction in her cloaca (possibly an undeveloped egg) and died after birthing 3 stillborns. Upon dissection, we discovered another 23 dead offspring within her. These offspring were weighed, but could not be accurately measured or sexed due to decomposition. Thus, this female was excluded from all analyses that utilized offspring SVL and/or sex as well as analyses of mortality.

5.2.4. Male Dissection and Sperm Counts

Male garter snakes were randomly allocated to aquaria such that treatment and control animals were housed together (6 males per aquarium; 8 aquaria total). In this species, the process of spermatogenesis occurs throughout summer and into the autumn (Krohmer et al. 1987). Consequently, we continued LPS injections of males throughout this period with the last injection given on 13 November. Males were euthanized in December and dissected to determine the masses of the testes and visceral fat bodies (± 0.001 g, A&D Fx-300i balance), and to collect sperm. For sperm collection, the ductus deferentia were carefully removed with fine forceps under a stereomicroscope (Nikon SMZ-745T) and placed in a 1.5 ml microcentrifuge tube containing 1 ml of sperm washing medium (Irvine Scientific) until the dissection was completed. The ductus deferentia and medium were then transferred to a small Petri dish containing an additional 2 ml of sperm washing medium. Again using the stereomicroscope, sperm were extruded from the ducts by holding the tissue with fine forceps while squeezing another pair of forceps along a section of the duct’s length.
Masses of sperm could readily be seen extruding from the open end of the duct and the procedure was continued until no more sperm was observed in this manner. The medium containing the sperm was pipetted into a 4 ml culture tube which was then centrifuged for approximately 5 minutes. The supernatant was removed and the sperm pellet was resuspended in 200 μl of 5% formalin to preserve the sperm for later counting. To obtain sperm counts we used methods similar to (Friesen et al. 2013). Briefly, each sample was warmed in a bead bath (30 °C) and gently shaken; a 4 ul aliquot was removed and placed on a Petroff-Hauser counting chamber at 10x magnification under an Olympus CX31 phase contrast compound microscope equipped with an Olympus DP-21 digital camera and CellSens software (Olympus). Samples that were overly concentrated (> 100 sperm on the counting grid) and that had clumped sperm were diluted to an appropriate concentration with glacial acetic acid. The sample was then photographed and saved in JPEG format. This procedure was repeated for a total of three replicates per sample. Sperm counts were conducted from the JPEG images in the CellSens software.

5.2.5. Statistical Analyses

We used residuals from linear regressions of ln mass on ln snout-vent length as estimates of body condition (SVL; Shine and Mason 2005). Separate regressions were conducted for adult males, adult females, and offspring. Mass, snout-vent length (SVL), and body condition were compared between treatments using Student’s t-tests when data were normally distributed and Mann-Whitney Rank Sum tests when data
did not meet normality. Feeding data were analyzed separately for males and females. A two-way ANOVA was used to assess treatment effects on food intake for gravid and non-gravid females. Treatment effects on male food intake were assessed with Student’s $t$-tests. Overall weight change was compared between LPS-treated and control snakes using a Student’s $t$-test for female data while male weight change data was assessed with Mann-Whitney rank sum tests due to non-normality (total males) and unequal variance (total males and the subset that were dissected). ANCOVA with female SVL as the covariate was used to assess treatment effects on litter mass and the number of offspring per litter; if there was significant interaction between the factor and covariate, the Johnson-Neyman procedure was used (Johnson and Neyman 1936; White 2003; Engqvist 2005). Specifically, the Johnson-Neyman procedure determines the boundaries of the region of non-significance, which contains the range of X-values for which treatment groups are not significantly different (Johnson and Neyman 1936; White 2003). To avoid pseudoreplication, we calculated average offspring mass, SVL, and body condition values for each litter and then compared the litters of LPS-treated and control females using Student’s $t$-tests or Mann-Whitney Rank Sum tests. The Johnson-Neyman procedure was carried out using an Excel spreadsheet designed by White (2003). All other statistical analyses were carried out using either SigmaPlot (version 12.5, Systat Software, Inc.) or XLStat (version 2013.4.8, Addinsoft). The box plot (Figure 5.2) was created with the online BoxPlotR application (Spitzer et al. 2014) which is available online from http://boxplot.tyerslab.com. All other figures were created in SigmaPlot.
5.3 Results

At the beginning of the experiment, within each sex, treatment and control groups did not differ in mass (Mann-Whitney Rank Sum; males: \( U = 274.0, P = 0.781 \); females: \( U = 306.50, P = 0.915 \)), snout-vent-length (SVL; Student’s t-test; males: \( t_{46} = -0.067, P = 0.947 \); Mann-Whitney Rank Sum: females: \( U = 307.50, P = 0.930 \)), or body condition (Student’s t-tests, males: \( t_{46} = 0.435, P = 0.665 \); females: \( t_{48} = 0.056, P = 0.955 \)).

5.3.1. Food Intake

During the ten weeks that food intake was monitored, 7 of 98 snakes (7.1%) died (four males and three non-gravid females), all of which were LPS-treated; the difference in mortality between the LPS (14.3%) and control (0%) treatments was significant (Fisher Exact Test, \( P = 0.006 \)). Because we accumulated 4-9 weeks of data for all of the snakes that died, we retained these snakes in our analyses. For females, food intake (mean % body mass consumed per week) was analyzed using a two-way ANOVA with treatment (control, LPS) and status (gravid, non-gravid) as factors. The analysis demonstrated a significant treatment by status interaction (\( F_{1,46} = 8.913, P = 0.005 \)) while the main effects were not significant (\( F_{1,46} = 0.398, P = 0.531 \) and \( F_{1,46} = 0.026, P = 0.872 \) for treatment and status, respectively). Bonferroni post-hoc tests revealed that mean food intake was significantly lower for non-gravid females treated with LPS (9.5% body mass eaten) compared to non-gravid females in
the control group (17.7% eaten; \( t = 2.791, P = 0.008 \), Figure 5.1a). However, there was no significant difference in food intake between treatments within the gravid female \( (t = 1.545, P = 0.129) \). Within the LPS treatment group, gravid females had higher food intake compared to non-gravid females \( (t = 2.211, P = 0.032) \) while within the control group gravid females tended to eat less than non-gravid females \( (t = 2.010, P = 0.050; \) Figure 5.1a). For males overall, there was no difference in food intake between LPS-treated and control groups (Student’s t-test, \( t = -0.493, P = 0.624; \) Figure 5.1b).

Considering overall change in body mass (i.e. week 1 pre-feeding mass as a percentage of their final pre-feeding mass), there was no significant treatment effect within either non-gravid females (Student’s t-test, \( t = 1.1596, P = 0.122; \) Figure 5.2) or total males (\( U = 279.000, P = 0.861; \) Figure 5.2). We did not assess the overall change in body mass for gravid females since this was confounded by their giving birth during weeks 8-10.

5.2.2. Gravid Females and Birth Data

Out of 50 total females, 42% (21 females) gave birth during the month of August (\( N = 10 \) LPS-treated, \( N = 11 \) controls). The proportion of females giving birth did not differ between the treatments \( (X^2, \text{d.f.} = 1, P = 1.000) \). There were no differences in mass (Mann-Whitney Rank Sum, \( U = 268.000, P = 0.603 \)), SVL (Mann-Whitney Rank Sum, \( U = 271.500, P = 0.653 \)), or body condition (Student’s t-
test, $t_{48} = -0.653, P = 0.514$) between females that gave birth and those that did not. One LPS-treated and one control female each produced two undeveloped eggs as part of their litters; these eggs were excluded from analyses of average offspring size and condition. However, as in Gregory (2006) we included the undeveloped eggs in our analyses of offspring number and litter mass, except where noted otherwise. Total litter mass was positively correlated with the number of offspring per litter (Linear Regression, adj. $R^2 = 0.804, P < 0.001$) while the average offspring mass per litter was negatively correlated with number of fully formed offspring (Linear Regression, adj. $R^2 = 0.583, P < 0.001$). After accounting for female SVL, the total number of offspring per litter was higher for LPS-treated females compared to controls (ANCOVA with female SVL as a covariate, $P = 0.028$, Figure 5.3a; this model excluded the SVL by treatment interaction which was non-significant ($P = 0.132$) in the full model). There was also a treatment effect on total litter mass as evidenced by a significant female SVL (covariate) by treatment interaction in an ANCOVA ($P = 0.023$) which demonstrated that the treatment effect varied with female SVL (Figure 5.3b). Further investigation using the Johnson-Neyman technique revealed that for females less than 63.6 cm SVL (this included 61.9% of females) those that were LPS-treated had greater total litter masses compared to controls. Regardless of treatment, the percentage of body mass consumed per week during pregnancy was not correlated with residuals from a regression of total litter mass on female SVL (Linear regression, adj. $R^2 = -0.052, P = 0.950$), but food consumption was positively correlated with residuals of ln post-partum mass on SVL (Linear regression, adj. $R^2 = 0.645, P < 0.001$). Post-partum mass, which was considered to be the pre-feeding mass of a
female at the weekly weighing immediately following giving birth, did not differ between LPS-treated and control females (Student’s t-test, $t_{16} = 0.622$, $P = 0.542$). Post-partum mass was not available for the three females with the latest births.

When considering only live offspring, neither the number of offspring per litter nor litter mass differed between treatments (ANCOVA with female SVL as covariate; $P = 0.116$ and 0.093, respectively; in both instances the non-significant ($P > 0.05$) covariate by treatment interaction terms were excluded from the final model). The percentage of offspring in a litter that were stillborn did not differ between treatments (Mann-Whitney Rank Sum, $U = 35.000$, $P = 0.272$). Per litter means of offspring mass, SVL, body condition, and sex ratio also did not differ between LPS-treated and control females (Student’s t-tests, $P > 0.200$ for all; Table 5.1).

Offspring mortality, measured as the percentage of offspring per litter that died by 60 days post-birth, was higher for litters from LPS-treated females (Student’s t-test, $t_{18} = 2.157$, $P = 0.044$). For the LPS-treatment, 3 of 9 litters (33.3%) had no offspring surviving to day 60 while only 1 of 11 litters (9.1%) from the control group had no surviving offspring; however, this difference was not significant (Fisher exact test, $P = 0.285$). We chose to assess mortality at 60 days as this time point coincided with the time of year when freezing temperatures become frequent in Manitoba and red-sided garter snakes return to hibernacula.
5.2.3. Male Dissections and Sperm Counts

A total of thirty-five (N = 20 control males, N = 15 LPS-treated) out of the original forty-eight males survived until the time of dissection in December (~6 months after the first injections); survival did not differ significantly between the treatments ($X^2 = 1.688$, d.f. = 1, $P = 0.194$). For these thirty-five males, although body condition did not differ between treatments at the beginning of the experiment (Student’s t-test, $t = -0.069$, $P = 0.946$), at the time of dissection body condition was higher for the LPS-treated males (Student’s t-test, $t = -0.767$, $P = 0.020$). Based on this finding regarding body condition, we revisited our feeding data set and analyzed food intake and mass change data for only the thirty-five males surviving until December. For these males, LPS-treated individuals consumed a greater percentage of their body weight each week compared to control males (Student’s t-test, $t = -2.137$, $P = 0.040$, Figure 5.1b). Overall body mass changes (week 1 pre-feeding mass as a proportion of their final pre-feeding mass) also differed significantly between treatments with LPS-treated males having a median final mass that was 131.3% of their week 1 mass compared to control males whose final mass was 98.6% of their week 1 mass (Mann-Whitney Rank Sum, $U = 86.000$, $P = 0.034$; Figure 5.2). Thus, LPS-treated males apparently increased in mass during the experiment whereas control males maintained their mass. For the following ANCOVAs, male body mass was included as a covariate; each model was first run with a treatment by covariate interaction term which was found to be non-significant in all cases ($P > 0.05$) and so excluded from the model presented below. The mass of visceral fat bodies (natural-
log transformed to correct non-normal residuals) did not differ significantly between treatments (ANCOVA, \( P = 0.272 \); Figure 5.4a). However, liver mass was significantly greater for LPS-treated males (ANCOVA, \( P = 0.027 \); Figure 5.4b). There was no difference in testes mass between treatments (ANCOVA, \( P = 0.238 \)) and square root transformed sperm counts also did not differ (ANCOVA, \( P = 0.235 \)).

### 5.4 Discussion

The results of our experiment did not clearly demonstrate tradeoffs between the immune and reproductive systems. The LPS-immune challenge did not affect females propensity to give birth, nor did it affect the proportion of stillborn offspring or individual offspring size. However, there was evidence that longer term offspring survival was negatively affected by LPS-treatment. For testes masses and sperm counts, we found no evidence of reproductive suppression in response to the immune challenge. The apparent lack of immune-reproduction tradeoffs, however, may be due to the fact that resources were not limited in our study (French et al. 2007a). That is, garter snakes may be able to facultatively regulate the tradeoff between the immune and reproductive systems based on resource availability (French et al. 2007a). As food resources may be more limited for wild snakes, particularly in drier years since frogs are a major dietary component (Gregory and Stewart 1975), future studies restricting food intake during immune challenges would be informative. For wild snakes, in times of plentiful resources, increasing food consumption to mitigate a bacterial immune challenge could come with additional costs such as increased risk of
predation, or the trophic acquisition of a greater number of macroparasites (e.g. trematodes, *Alaria* spp.) which can have their own adverse effect on the snakes’ fitness (Uhrig et al., chapter 2 of this dissertation).

Anorexia is a common feature of the sickness response that occurs during an endotoxin-mediated immune challenge (Grunfeld et al. 1996; Exton 1997). Although somewhat counterintuitive, reduced food intake has been shown to improve survival of infected individuals (Wing and Young 1980; Exton 1997). Indeed, some components of the immune system (e.g. macrophage and natural killer cell activity) are enhanced by fasting (Wing et al. 1983; Exton 1997). However, in our study a reduction in food intake by LPS-treated individuals was only observed for the non-gravid females. Although not statistically significant, gravid females treated with LPS tended to eat more than controls while, overall, males in the two treatment groups consumed nearly equal amounts. If the repeated immune challenges were indeed perceived as a threat to survival (Kivleniece et al. 2010), then the observed patterns of food intake may represent different optimal strategies for maximizing lifetime reproduction. The non-gravid females, having already foregone the opportunity to produce offspring during the present breeding season, may have benefitted most from utilizing an anorexic strategy to help overcome the perceived infection and increase chances for survival to the following breeding season. Although the anorexia would have impeded the acquisition of resources for overwinter survival, the difference in mass change over the course of the summer between LPS-treated and control non-gravid females was negligible. That the LPS-treated non-gravid females did not
greatly lose mass despite their anorexia could be due to lower activity levels, which is also a common part of the sickness response (Owen-Ashley and Wingfield 2007), but this was not tested in the current study.

Females that became gravid were likely undergoing vitellogenesis and ovulation prior to the first LPS injection in June as these reproductive events occur within approximately 6 weeks following emergence (Whittier et al. 1987). Therefore, having already committed to investing in reproducing, these females may have benefitted most from maintaining or even elevating food intake in a strategy to increase the likelihood of successfully producing offspring during the current season. Previous studies have shown that gravid female garter snakes typically eat less than non-gravid females (Gregory et al. 1999; Gregory 2001), which is the trend, albeit non-significant, that we observed within the control treatment. However, within the LPS-treatment the trend was reversed and gravid females exhibited higher food consumption which could indicate compensation for the immune challenge and increased investment in reproduction. In support of increased reproductive investment, we found that after accounting for differences in female size, LPS-treated females produced more offspring per litter and, at least for the relatively smaller females, litter masses were greater for LPS-treated females, which is indicative of increased investment in offspring production. Although offspring mortality was higher for LPS-treated females, two-thirds of these females still had at least one offspring survive to 60 days post-birth. Despite the tendency for LPS females to have both higher litter mass and increased food intake, in accordance with Gregory (2006)
we found post-partum mass, but not litter mass, to be positively correlated with food intake during pregnancy. That is, litter mass appears to be based more on capital (i.e. fat stores) than on income (food intake during the summer). Therefore, if LPS-treated females increase investment in offspring production, they are likely doing so by adjusting the allocation of stored resources. In contrast, the increased food intake by LPS-treated gravid females may be necessary to compensate for the immune challenge while supporting increased metabolic demands of pregnancy and maintaining post-partum mass to increase overwinter survival (Gregory 2006).

Indeed, post-partum mass did not differ between LPS-treated and control females. However, any female overcoming an immune challenge during the feeding season and surviving hibernation may nonetheless face a cost to their reproductive fitness in the following year if their ability to accumulate sufficient capital was compromised. Future studies, therefore, should examine the longer term consequences of immune challenges in garter snakes as reproductive fitness costs may only manifest in the subsequent breeding season.

The males in our experiment also showed no indication of LPS-induced anorexia. During late summer and early autumn, testicular activity reaches its peak as males prepare their sperm stores for the following mating season (Krohmer et al. 1987). Additionally, as female garter snakes often produce offspring from sperm stored over hibernation (Halpert et al 1982; Friesen et al. 2014), by mating during the autumn period, a male can increase his chances of siring offspring the following year even if he does not survive the winter (Friesen et al. 2014). Thus, the absence of
anorexia in males may represent a strategy for maintaining resources to facilitate autumn matings and their potentially posthumous fitness benefits (e.g. López-Sepulcre et al. 2013). That we found no differences in testes masses or sperm counts between LPS and control males indicates males were also apparently able to maintain sperm production when immune challenged, which would be crucial to furthering fitness should he survive the winter. However, as previously mentioned, the absence of a tradeoff may be due to the availability of resources. Indeed, when considering only the males from which we obtained the testes mass and sperm count data, those treated with LPS actually consumed more food compared to control males which may represent their compensating for the immune challenge by increasing food intake as was observed by Tyler et al. (2006) in bumble bees (*Bombus terrestris*).

Interestingly, for the males that were dissected, those treated with LPS also showed a significant increase in mass over the summer compared to control males. However, this mass gain was not in the form of increased visceral fat stores as these did not differ significantly between treatments. Rather, we observed a significantly increased liver mass for the LPS-treated males compared to the controls. In general, elevated liver mass could indicate increased glycogen or lipid storage which would be beneficial during hibernation and the subsequent breeding season (Aleksiuk and Stewart 1971; Costanzo 1985). However, given the immunological role of the liver particularly in clearing endotoxins like LPS (Jirillo et al. 2002; Bilzer et al. 2006; Gao et al. 2007), it is more likely that the increased liver mass represents part of the detoxification response. Indeed, several studies have demonstrated increased liver
mass in response to LPS treatment (Qian and Brosnan 1996; Best et al. 2003; Cani et al. 2007; Shao et al. 2011). In the long term, elevated liver mass induced by the presence of endotoxin could be indicative of immunopathology where excessive or prolonged activation of the immune system can be damaging independent of any direct effects of a pathogen (Bone-Larson et al. 2000; Graham et al. 2005; Sadd and Siva-Jothy 2006). In the case of liver immunopathology, prolonged LPS-induced inflammation and elevated cytokine levels could, for example, lead to nonalcoholic fatty liver disease where accumulation of triglycerides results in liver damage (Baffy 2009; Valenti et al. 2009). For garter snakes, and indeed for most non-rodent taxa, the possibility of LPS-induced liver damage has not been explored yet it clearly has potential fitness effects if liver function is compromised (e.g. altered nutrient storage or detoxification ability). Thus, our finding of increased liver mass in response to LPS highlights the importance of considering immunopathology as a resource-independent cost of immune activation that could affect reproductive fitness.

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5.5 References


Table 5.1. Mean (± SE) values per litter for offspring mass, snout-vent length (SVL), body condition, and sex ratio from litters of red-sided garter snakes where mothers received either lipopolysaccharide (LPS) or saline (control) injections. Mass data shown are for litters from N = 10 LPS-treated, N = 11 control females. Offspring from one LPS litter could not be accurately sexed or measured and so were excluded from analyses of SVL, body condition, and sex ratio (i.e. N = 9 LPS-treated, N = 11 control for these analyses).

<table>
<thead>
<tr>
<th>Treatment Group of Mother</th>
<th>Offspring Mass (g)</th>
<th>Offspring Snout-vent Length (cm)</th>
<th>Offspring Body Condition (residuals from regression of ln mass on ln SVL)</th>
<th>Sex Ratio (male proportion of offspring)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>1.042 ± 0.079</td>
<td>14.475 ± 0.337</td>
<td>-0.038 ± 0.161</td>
<td>0.540 ± 0.039</td>
</tr>
<tr>
<td>Control</td>
<td>1.165 ± 0.060</td>
<td>14.712 ± 0.285</td>
<td>0.221 ± 0.170</td>
<td>0.488 ± 0.045</td>
</tr>
<tr>
<td><strong>Statistical Analysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Student’s t-test)</td>
<td>t_{19} = 1.260</td>
<td>t_{18} = 0.540</td>
<td>t_{18} = 1.083</td>
<td>t_{18} = -0.842</td>
</tr>
<tr>
<td></td>
<td>P = 0.223</td>
<td>P = 0.596</td>
<td>P = 0.293</td>
<td>P = 0.411</td>
</tr>
</tbody>
</table>
Figure 5.1. Mean food intake (± SE) measured as percent body mass consumed per week over the course of ten weeks for red-sided garter snakes treated with either lipopolysaccharide (LPS) or saline (control). A) non-gravid and gravid females and B) total males and dissected males; the dissected males are a subset of the total males. Asterisks denote significance at the $\alpha \leq 0.05$ level.
Figure 5.2. Change in body mass measured as percent change from first pre-feed weighing to final pre-feed weighing for red-sided garter snakes treated with either lipopolysaccharide (LPS) or saline (control). The dissected males are a subset of the total males. The limits of the boxes correspond to 25th and 75th percentiles while whiskers extend 1.5 times the interquartile range from these percentiles (determined by R software); medians and means are represented by solid center lines and crosses, respectively. Asterisks denote significance at the $\alpha \leq 0.05$ level.
Figure 5.3. A) Number of offspring per litter (ANCOVA, main effect of treatment: $P = 0.028$) and B) total litter mass (ANCOVA, covariate by treatment interaction: $P = 0.023$) in relation to female snout-vent length (SVL) for female red-sided garter snakes injected with either lipopolysaccharide (LPS) or saline (control). For panel B, the vertical dotted line indicates the lower boundary (63.6 cm) of the region of non-significance based on the Johnson-Neyman procedure; in the region to the left of the dotted line, total litter mass differs significantly between treatments. The upper boundary (90.8 cm) of the region of non-significance is not shown.
Figure 5.4. A) Ln visceral fat mass (ANCOVA, main effect of treatment: $P = 0.272$) and B) liver mass (ANCOVA, main effect of treatment: $P = 0.027$) in relation to total body mass for male red-sided garter snakes injected with either lipopolysaccharide (LPS) or saline (control).
Chapter 6: General Discussion

In this dissertation I have investigated parasitic infections and immune challenges in garter snakes. My results provide evidence linking parasites and immune responses to host reproductive fitness. Further, my results illustrate that the reproductive fitness effects of infections and immune responses can occur in a variety of context dependent ways even within a single host species. Thus, my work underscores the importance that studies of host-parasite relationships consider not just a single parasite or single fitness correlate when investigating potential parasite-mediated fitness consequences.

In chapter 2, I took a histopathological approach to assessing the consequences of infection by the trematode *Alaria*. In this instance my results provide evidence for the presence of parasites mechanically damaging host tissues in a manner that is likely to adversely affect reproductive fitness. The large numbers of *Alaria* mesocercariae that aggregate in the snakes’ tails lead to mucus accumulation and damage of the tail musculature which likely increases the propensity for tail loss. The loss of the tail has negative fitness consequences for reproduction in both male and female hosts (Pisani 1976; Perry-Richardson et al. 1990; Shine et al. 1999). Thus, my results demonstrate an interesting, and somewhat uncommon, scenario of a parasite compromising host fitness via mechanical impairment. Future studies should be aimed at determining whether the extent of tail damage is mediated primarily by parasite density or by variation in host tolerance to the presence of *Alaria*. The accumulated mucus surrounding the parasites is likely of host origin, but additional studies are needed to confirm this. If indeed the mucus is host-derived, then this
provides evidence that the host response to the parasites’ presence may mediate the severity of the tissue damage.

In chapter 3, I broadened my investigation to include more of the helminth parasites typically present in red-sided garter snakes and plains garter snakes in Manitoba. My results characterize interspecific and interpopulational variation in the helminth assemblages in terms of parasite prevalence and density providing a useful foundation for future studies. Importantly, I found that helminth densities are predictive of host attributes that have direct relevance to male fitness, namely body condition, testes mass, and sperm counts. To the best of my knowledge, this is the first study to provide evidence linking parasites and host fitness correlates in a garter snake and one of only a few such studies of snakes in general. Although interactions between helminths were not significantly predictive, my results indicate additive effects between helminths and show that different types of helminths are predictive of different fitness correlates. Thus, my results emphasize the importance of considering multiple parasites when assessing the fitness consequences of parasitism in garter snakes.

Interestingly, helminth densities were predictive of body condition, testes mass, and sperm counts in only one of the two red-sided garter snake populations examined. This reason for this difference between the populations is not clear as helminth densities were quite high in both populations. The Inwood population, in which helminths were not predictive of fitness correlates, actually had significantly higher density of *Alaria* which was a significant predictor of all the fitness correlates in the other red-sided garter snake population (Snake Island). The Inwood population,
however, is much larger and less isolated compared to Snake Island (Mason et al. 1991; Shine et al. 2006) which could result in selection pressures acting differently in the two populations (Frankham 1996; Gandon and Michalakis 2002; Charlesworth 2009), but this will need to be more thoroughly investigated. Additional comparisons of parasitism and fitness correlates between other garter snake populations of varying sizes would be informative.

Notably, the results of both chapters 2 and 3 provide some evidence that variation in parasite resistance may be present among garter snakes. All of the parasites we examined are trophically transmitted and, generally, it could be expected that larger snakes would consume more or larger prey and so ingest more parasites. However, in both studies, my results indicate that larger bodied snakes were not necessarily the most heavily infected with a given parasite. Given that a single frog, typical prey of garter snakes, can be infected with thousands of helminths (Fernandes et al. 1976) and that a snake will eat many such infected frogs in its lifetime, clearly not every ingested parasite becomes established within the snake. Thus, snakes must have some basic degree of resistance to the establishment of parasites and/or the ability to clear parasites over time. If snakes vary in their levels of resistance to parasites, then such variation could explain the observation that the largest snakes did not always have the highest parasite burdens. Alternatively, the lack of correlations between snake size and parasite burden could result simply from differences in diet. Thus, future studies are needed to investigate the possibility of variation in parasite resistance. An experimental design in which uninfected snakes of different populations or species are fed a specific number of parasites and then examined for
parasite establishment in their tissues would be particularly useful. However, such a design presents some logistical difficulties due to most of the focal helminths having 100% prevalence (i.e. all wild snakes are infected). There are at least two ways in which to overcome this difficulty. First, infected snakes could be treated with anti-parasitic drugs (e.g. ivermectin and praziquantel). However, it may not be possible to entirely eliminate high density infections in this manner. Second, if the parasites are not vertically transmitted from mother to offspring, wild caught mated females from different populations or species could be allowed to give birth in the laboratory and their parasite-free offspring reared in captivity; this approach has the added advantage of creating a common-garden environment for subsequent experiments.

In chapters 4 and 5, I used a bacterial-derived endotoxin, lipopolysaccharide (LPS), as an immune challenge in order to assess immune-reproduction tradeoffs. Eliciting an immune response in this manner, that is, based on a perceived bacterial invasion, is not a marked departure from the parasite studies of the preceding chapters as bacteria are considered microparasites (e.g. Anderson and May 1979). It is, however, worth noting that the exact nature of the immune response to a bacterial-derived challenge may not be precisely the same as that elicited by macroparasites like helminths (e.g. Jankovic et al. 2001; Zimmerman et al. 2010). Nonetheless, the use of LPS, which is non-pathogenic itself, serves as a useful starting point for exploring the reproductive implications of immune activation independent of the pathogenic effects of a parasite.

In chapter 4, my results demonstrated that, during the breeding season, an immune challenge adversely affected male reproductive investment. Male courtship
and mating success were significantly reduced and, for males that did mate, copulatory plug masses were lower for LPS-treated males compared to control males. Sperm counts, however, did not differ between treatments which is not unexpected because, as dissociated breeders, red-sided garter snakes complete spermatogenesis in the autumn preceding spring breeding. The observed reproductive tradeoffs appear to be hormonally-mediated as LPS-treated males exhibited substantially increased plasma corticosterone levels and decreased androgen levels compared to control animals. With male garter snakes having relatively low androgen levels during the breeding season, it may be that testosterone-mediated immunosuppression was relaxed allowing for an immune response, but it is currently unclear as to whether such immunosuppression occurs in this species. Future studies utilizing hormonal treatments and including quantification of immune responses would be informative to further elucidate immune-endocrine interactions in these snakes. The results of this study highlight the influence of host life history on the consequences of immune activation and also emphasize the complex interactions between the immune, reproductive and endocrine systems.

In chapter 5, where snakes received repeated immune challenges throughout the summer, immune-reproductive tradeoffs were much less apparent despite this period of time encompassing important reproductive events such as females giving birth and males undergoing spermatogenesis. However, the experiment was conducted in the context of ample food resources and our results regarding food intake indicate that males and gravid females compensated for the immune challenge by increasing their food consumption. Future studies should administer immune
challenges under regimes of limited food availability to determine whether tradeoffs become apparent in such circumstances. Additionally, as garter snakes are capital breeders with a hibernation period intervening between the feeding and mating season, investigations of the longer term consequences of immune challenges would be informative. That is, an immune challenge that occurs during the summer feeding period and alters food intake, and thus resource accumulation (i.e. acquisition of capital), may have consequences for overwinter survival, mating success during the following spring, and/or reproductive processes during the following summer. Also worthy of future investigation, is the possibility of immunopathological effects of prolonged immune activity. LPS-treated males, for example, increased food intake and actually exhibited body mass gains, but this appeared to occur via increased liver mass which may be indicative of liver damage induced by prolonged immune activation.

As a whole, the work presented in my dissertation demonstrates that parasitic infections and immune responses do indeed have reproductive fitness implications for garter snakes. Further, my work provides a valuable foundation on which future studies can be based. With their well-studied physiology and behavior, garter snakes are particularly useful models for broadening our understanding of host-parasite relationships and associated immune responses in wild taxa and will help elucidate parasite- and immune-mediated selection processes in reptiles, which may, more broadly, contribute to a better understanding of the evolution of the vertebrate immune system (e.g. Zimmerman et al. 2010). The importance of investigating snake parasites and their fitness consequence is further highlighted by snakes’ susceptibility
to emerging infectious diseases such as the fungal infection caused by *Ophidiomyces ophiodiicola* (formerly *Chrysosporium*) that has been found infecting wild snakes, including *Thamnophis* spp. (Dolinski et al. 2014), in the United States (Nichols et al. 1999; Allender et al. 2011; Latney and Wellehan 2013; Mitchell and Walden 2013; Sleeman 2013). It is unclear how parasitic infections may interact with this fungal pathogen, but investigations of parasitic infections are crucial if we are to fully understand the dynamics of emerging infectious diseases and interactions among pathogens within snakes.

6.1 References:


Bibliography:


