AN ABSTRACT OF THE THESIS OF

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Male Red-sided Garter Snake, *Thamnophis sirtalis parietalis.*</u>

Abstract approved: _____

Robert T. Mason

The benefits of reproduction are clear, but there are also costs. Much is
known about the costs of reproduction in females, but only recently have male costs
been investigated in any depth. These costs of reproduction may be minimized by
appropriately modifying behavior, but there has been little research on such
behavioral minimization, especially in males.

Male red-sided garter snakes, *Thamnophis sirtalis parietalis*, are an excellent
model for the study of reproductive behavior because they form large breeding
aggregations and tolerate human observation and manipulation. They are
particularly useful for the study of reproductive costs because their investment in
reproduction ends at copulation, and thus all reproductive effort is spatially and
temporally constrained to the breeding sites during the breeding season.

We investigated two ways in which male garter snakes use behavior to
minimize reproductive costs. First, we investigated whether male garter snakes can
discriminate between the pheromone trails of mated and unmated females. After

mating, females are unable to remate for two to four days due to a mating plug that occludes their cloaca. As males search for females by following their pheromone trails, they are exposed to high costs in terms of energetics, risk of predation, and lost opportunities with other females. Males discriminated between the pheromone trails of mated and unmated females, and they preferentially followed the trails of unmated females. They followed mated females once the mating plug had disintegrated and the female was able to remate. These results suggest that by discriminating between the trails of mated and unmated females, males can minimize the costs associated with searching for females by preferentially following females that provide a mating opportunity over those with which mating is not possible.

Second, we investigated seasonal anorexia in male garter snakes. Despite high daily energy expenditure in the breeding season and eight months of aphagia preceding the breeding season, males are never found with prey items in their stomachs at the dens. This aphagia has been attributed to extrinsic causes (a lack of prey at the dens) and to intrinsic causes (seasonal anorexia). We determined that males at the breeding grounds exhibit a seasonal anorexia, whereby they show no interest in feeding during the breeding season. This anorexia appears to be adaptive in light of the high cost of searching for prey during the breeding season.

We conclude that male garter snakes adjust their behavior to minimize the costs of reproduction. However, at this point our understanding of the nature of these costs is primarily qualitative. Future work should expand our knowledge of the costs associated with reproduction, especially in quantifying costs experienced by the male. Historically these costs have been neglected.

Behavioral Adaptations and the Minimization of Reproductive Costs in the Male
Red-sided Garter Snake, *Thamnophis sirtalis parietalis*

by

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

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Ryan P. O'Donnell, Author

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CONTRIBUTION OF AUTHORS

Robert T. Mason served as my graduate advisor, and all of the work of this thesis was done with his guidance and much of his funding. Richard Shine contributed to experimental design and provided critical discussion during data collection for Chapters 2 and 3. Neil B. Ford provided the initial idea for Chapter 2 and assisted with data collection. All authors assisted in reviewing and editing Chapters 2 and 3.

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CHAPTER I

GENERAL INTRODUCTION

Costs of Reproduction

The benefits of reproduction are clear. Reproduction is the process of generating offspring, which are the measure of fitness. Only through reproduction can an animal increase the frequency of its genes in a population. But there are also costs involved in reproduction. These costs of reproduction are key to the understanding of many fundamental concepts in biology, including sexual selection, the evolution of sexual reproduction, and life history evolution (Darwin, 1874; Trivers, 1972; Ghiselin, 1974; Andersson, 1994; Schwarzkopf, 1994).

The costs of reproduction, called "reproductive effort" by some authors (*e.g.* Low, 1978; Caley *et al.*, 2001) can be divided into two classes: the costs of finding and securing a mate, or mating effort, and the costs of producing offspring, or parental effort (Low, 1978; Simmons and Parker, 1989). Mating effort is rewarded with copulations, while parental effort is rewarded with offspring.

While males and females always incur some cost of reproduction, the distribution and magnitude of these costs can vary dramatically between the sexes

(Trivers, 1972; Thornhill, 1976; Trivers, 1985). Usually, but not always, the cost of

reproduction for males is primarily composed of mating effort, while in females it is

primarily parental effort (Ridley, 1978; Zeh and Smith, 1985).

Costs in Females

Although females tend to expend more in reproductive effort than mating

effort, they do experience some costs attributable to mating effort. For example,

courtship by males imposes heavy survival costs in some species. In garter snakes,

harassment by males imposes energetic costs on females and increases their risk of

predation (Shine *et al.*, 2000a; Shine *et al.*, 2003a; Shine *et al.*, in press).

Reproduction can be very costly in terms of exposure to predators. Courting males

of the orb-web spider, *Argiope keyserlingi*, attract predatory mantises to the female

spiders' webs and thus increase the risk of predation for both the male and female

spiders (Herberstein *et al.*, 2002). Courting pipefish experience a higher risk of

predation by leaving an area of cover (Berglund *et al.*, 1986b). Breeding in females

is sometimes associated with a period of fasting, especially in species such as green

sea turtles, *Chelonia mydas*, and southern elephant seals, *Mirounga leonine*, that

forage in water but come to shore to reproduce (Fedak *et al.*, 1994; Tucker and Read,

2001). Male courtship can also directly restrict female feeding, as in the water

strider *Gerris buenoi*, (Rowe, 1992; Ortigosa and Rowe, 2002). Finally, males force

copulation in some species, and such forced copulation can cause serious injury and

death (Thornhill, 1980; McKinney *et al.*, 1983; Le Boeuf and Mesnick, 1990; Robertson, 1990).

Courtship also has energetic costs in species where females must expend energy in evaluating and selecting among potential mates. For example, in lekking species, females must travel through the leks in order to evaluate males while avoiding harassment. To travel the female must expend energy (Bourne, 1993; Alatalo *et al.*, 1996). Finally, females sometimes expend energy actively courting males in "role-reversed" species such as dart-poison frogs, *Dendrobates* sp., and pipefish and seahorses of the family Syngnathidae, for example (Berglund *et al.*, 1986b; Summers, 1992). I will consider the energetic costs of courting below, in the context of the more typical courtship by males.

Costs to females are also incurred in copulation. For example, in fruit flies, *Drosophila melanogaster*, males transmit toxic substances in their semen which reduce female life span (Fowler and Partridge, 1989; Chapman *et al.*, 1995). Copulation can physically damage the female genitalia, which reduces the life span of the female bean weevil, *Callosobruchus maculatus* (Crudgington and Siva-Jothy, 2000). A cost of reproduction that is shared by both males and females is the risk of exposure to sexually or socially transmitted pathogens (Poiani and Wilks, 2000). In some species, reproduction involves lengthy copulation in which the male and female are both restricted from independent movement for hours or even days. For example, in the southern green stink bug, *Nezara virigidula*, copulation may last for seven days (Harries and Todd, 1980), and in the lygaeid bug *Lygaeus equestris*, it can last 12 days (Sillén-Tullberg, 1981).

Parental effort is more apparent, and more significant, than mating effort for many female animals. The cost of producing young begins with the production of gametes, which is by definition higher in females. But these gamete costs are often minor compared to the energetic investment that occurs after fertilization.

In females with internal fertilization, gestation imposes a physical and physiological burden. Gestation can subject the female to exposure to predators and reduced foraging. For example, pregnancy reduces running speed and endurance in many lizards (see references in Schwarzkopf, 1994). In addition, gestation can increase basking requirements and reduce feeding (Huey and Slatkin, 1976; Mrosovsky and Sherry, 1980; Shine, 1980; Schwarzkopf, 1994; Weeks, 1996; Gregory *et al.*, 1999; Lourdais *et al.*, 2002). These changes are often assumed to affect survival, and have occasionally been shown to do so (Shine, 1980; but see Bauwens and Thoen, 1981).

Some animals have a period of maternal care after birth or hatching. For many mammals, most birds, and some fish, this care is the most costly part of reproduction, and can involve months or years of investment. Maternal care is taken to an extreme in some spiders and one cricket, in which the mother sacrifices her body for the nourishment of her offspring (references in Polis, 1981).

Costs in Males

Costs of reproduction are much less well known for males, and have been historically overlooked, especially in non-monogamous species (Deutsch *et al.*, 1990; Schwarzkopf, 1994). Studies of reproductive costs have often considered only parental effort, which is usually much higher in females. Because research focused on parental effort, not mating effort, the common view was that, as a general rule, "females do all the investing, males do none of it" (Trivers, 1985, p. 207). Male costs are usually in mating effort, rather than parental effort.

Perhaps the most obvious, and sometimes the most severe, costs of reproduction for most males are the costs associated with courtship (Andersson, 1994). Ironically, the costs involved in male competition for females are so visually apparent that they have garnered less study than female choice among the competing males (Andersson, 1994). In many species, males compete with one another for access to females, and these competitions can be very costly in terms of developing and maintaining weapons (Goss, 1970), energetics of combat, and mortality (Geist, 1971; Clutton-Brock, 1982; Andrén, 1985). The risk of mortality may also be increased due to increased mobility and/or conspicuousness, and hence exposure to predators, parasites, and even automobile traffic during periods of courtship and breeding (Cade, 1975; Aleksiuk, 1977; Andrén, 1985; Lima and Dill, 1990; Bonnet and Naulleau, 1996). Many males expend vast amounts of energy in courtship displays and lose significant proportions of energy stores over the course of the

breeding season (Geist, 1971; Anderson and Fedak, 1985; Naulleau *et al.*, 1987; Deutsch *et al.*, 1990; Andersson, 1994; Bonnet and Naulleau, 1996).

These energetic losses are made even more consequential because many males reduce or even abandon feeding during the breeding season (Geist, 1971; Mrosovsky and Sherry, 1980; Anderson and Fedak, 1985; Robinson and Doyle, 1985; Robertson, 1986; Deutsch *et al.*, 1990; Fedak *et al.*, 1994; Bonnet and Naulleau, 1996). This lack of feeding may be due to an intense focus on breeding during the appropriate season, or it may be because feeding and breeding are mutually exclusive for other reasons such as the spatial and temporal distribution of mates and food. In addition to reducing energy stores, the lack of feeding due to courtship and mate guarding may also reduce male growth, as in the amphipod *Gammarus lawrencianus* (Robinson and Doyle, 1985). In fact, the ability to withstand long periods of aphagia may be a key component of sexual selection in some species (Bartholomew, 1970; Andersson, 1994).

Energy loss during the breeding season is also manifested in terms of material losses. Many species, especially among the insects (Thornhill, 1976; Rutowski, 1982), but also rodents (Baumgardner *et al.*, 1982; Koprowski, 1992), amphibians (*e.g.* Baker, 1990; Verrell and Mabry, 2003), and snakes (Devine, 1975; Shine *et al.*, 2000b), produce a copulatory plug or spermatophore that is transmitted at copulation, and can weigh up to 10% of the male's mass (Thornhill, 1976; Rutowski, 1982). Some animals lose body parts in mating. For example, the male black widow spider loses the tip of its palpal embolus (the copulatory organ) (Buskirk *et al.*, 1984). Some males provide nuptial gifts (prey items or, rarely, plant

material) that they have collected to present to the female in courtship (Calder, 1967; Thornhill, 1976 and references therein). In terrestrial arthropods, a nuptial gift is the most common type of male reproductive effort (Thornhill, 1976; Buskirk *et al.*, 1984; Zeh and Smith, 1985), and it is also a common part of bird courtship behavior (Pizzari, 2003). The collection or preparation of nuptial gifts may also expose males to predation (Thornhill, 1975). The production or acquisition of these substances may be the limiting factor for reproduction (Thornhill, 1976, 1980; Rutowski, 1982). The costs associated with nuptial gifts have been considered either mating effort or parental effort, depending on the degree of incorporation of the energy in the male's offspring (and on the author's interpretation of the selective pressures resulting in the evolution of the nuptial gift) (Gwynne, 1984; Simmons and Parker, 1989). The loss of material may include the ultimate reproductive cost, life, in the case of sexual cannibalism, as is seen in arachnids, insects, and copepods (*e.g.* Polis, 1981; Thornhill and Alcock, 1983; Buskirk *et al.*, 1984; Elgar and Fahey, 1996; Herberstein *et al.*, 2002; Schneider and Elgar, 2002). There is debate over the adaptive value of sexual cannibalism (*e.g.* Buskirk *et al.*, 1984; Liske and Davis, 1984; Simmons and Parker, 1989; Schneider and Elgar, 2002), but regardless of its adaptive value, it is clearly a reproductive cost.

The time spent in copulation can represent a significant cost for males, as for females. Missed opportunities for mating may be considered an additional cost of mating for the male, especially when females vary in quality (Parker, 1974). This opportunity cost can be a driving force in systems where mating opportunities are limited in time but not in number (Parker, 1974). The duration of copulation can

also induce costs in terms of risk of predation, as discussed above, but although male and female's time in copulation is equivalent, the risk to the two may not be. The individual on top (often the male) may be more susceptible to predation.

Some of the costs traditionally associated with female reproduction apply instead to males of certain species (Ridley, 1978; Trivers, 1985). For example, many male seahorses and pipefish bear the physical and/or physiological burdens associated with pregnancy (Berglund *et al.*, 1986a; Berglund *et al.*, 1986b; Wilson *et al.*, 2003), the costs of which have been discussed above. Males of some species also expend parental effort in the form of paternal care. In some taxa, males provide all of the parental care, where in others they share the duty with the female. Exclusively male parental care can be found in some polychaetes, hemipterans, amphibians, and birds, but is most common in pycnogonids and fish (Ridley, 1978; Wells, 1980; Gross and Shine, 1981; Berglund *et al.*, 1986b; Summers, 1990, 1992; García-González *et al.*, 2003). Amphibians and fish are somewhat anomalous among animals: in amphibians males account for half of parental care, and in fish male parental care is more common than maternal care (Gross and Shine, 1981). As a whole, however, paternal care is rather rare (Zeh and Smith, 1985). It has not been found in any squamate reptile (Shine, 1988).

Using Behavior to Minimize Costs

While there are many costs associated with reproduction, many of these costs may be minimized by using appropriate behavior. In addition, the relative magnitude of costs may be adjusted through behavioral modifications (Lourdais *et al.*, 2002). There has been very little research in this area, but at least four species are known to use behavior to reduce reproductive costs. For example, pregnant female common lizards, *Lacerta vivipara*, reduce the costs of increased predation due to decreased locomotor abilities by shifting to a more cryptic suite of predator avoidance behaviors (Bauwens and Thoen, 1981). Pregnant garter snakes, *Thamnophis ordinoides*, increase cryptic behavior, presumably to reduce the increased risk of predation during pregnancy (Brodie, 1989). Female bean weevils, *Callosobruchus maculatus*, reduce injury during copulation by kicking away mating males before they can inflict full damage to the female genitals (Crudgington and Siva-Jothy, 2000). The female brown tree snake, *Boiga irregularis*, releases a pheromone that inhibits unwanted male courtship (Greene and Mason, 2003).

While these studies show a behavioral reduction in the cost of reproduction, there is a need for more research on the ways in which animals adjust their behavior to reduce their reproductive costs (Brodie, 1989; Stearns, 1992). This need is especially acute for males, whose reproductive costs have been historically overlooked (Schwarzkopf, 1994) and who have not been shown to use behavior to minimize those costs.

Summary of Research Goals

The goals of this thesis are to explore two ways in which male red-sided garter snakes, *Thamnophis sirtalis parietalis*, use behavior to minimize the costs associated with reproduction. The life-history of the red-sided garter snake makes it an excellent species in which to address these questions. Their reproductive behavior is very robust and easily quantified. Also, many potential reproductive costs can be eliminated conclusively. Because of their short breeding season (4 weeks) and massive aggregations, there is a high cost of missed opportunities with other females (Gregory, 1974; Shine *et al.*, 2001a), and a high risk of falling prey to the predators that feed at the aggregations (Aleksiuk, 1977; Gregory, 1977b; Shine *et al.*, 2001b). Furthermore, we can eliminate from consideration any costs associated with parental care because male garter snakes, and male snakes in general, show no parental care for the young (Shine, 1988). Male investment in reproduction ends with copulation.

We investigated the behavioral minimization of reproductive costs in two contexts. First, we looked for a behavioral adaptation that would minimize the high costs (in terms of energetics, missed mating opportunities, and risk of predation) of searching for females. The cloacae of recently mated females are completely occluded by a copulatory plug, and thus these females are not able to remate until the plug disintegrates about three days later (Devine, 1977; Ross and Crews, 1977, 1978; Shine *et al.*, 2000b). Males follow pheromone trails to find females (Ford, 1981;

Ford and Low, 1984; Ford and O'Bleness, 1986; Mason, 1992; LeMaster *et al.*, 2001), and they can determine female mating status upon contact (Devine, 1977; Ross and Crews, 1977, 1978; Shine *et al.*, 2000b). This chapter investigates whether males can discriminate between mated and unmated females from these pheromone trails and thus minimize the costs of searching for females by avoiding potentially costly searches for females that are not potential mates.

Second, we investigated the ways in which the costs of reproducing and the costs of feeding are mediated behaviorally. Because of the short breeding season and the spatial distribution of prey and mates, there would be a very high reproductive cost associated with searching for food during the breeding season (Shine *et al.*, 2002). Despite enduring eight months of aphagia through winter (Gregory, 1977b), males emerge from hibernation and begin courting in the spring, yet are never found at the breeding grounds with prey in their stomachs (Aleksiuk and Stewart, 1971; Gregory and Stewart, 1975; Crews *et al.*, 1987). We investigated whether this aphagia is an artifact of the spatial distribution of mates and prey, or rather an intrinsic anorexia where males show no interest in food during the breeding season.

Finally, I conclude with a summary of our findings and a discussion of some potential areas for future work.

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CHAPTER II

MALE RED-SIDED GARTER SNAKES (*THAMNOPHIS SIRTALIS PARIETALIS)* DETERMINE FEMALE MATING STATUS FROM PHEROMONE TRAILS

Ryan P. O'Donnell, Neil B. Ford, Richard Shine, and Robert T. Mason

Abstract

Recently mated females of some species experience a refractory period following mating during which a physiological change or a mating plug prevents remating. Males that determine female mating status from a distance will minimize time spent searching for mates by avoiding unavailable females. Female red-sided garter snakes experience a refractory period after mating due to a mating plug. Female garter snakes leave pheromone trails, which males detect and follow to find potential mates. We investigated the ability of male garter snakes to determine the mating status of females based solely on these pheromone trails. Males were given a choice between following two trails on a Y-maze to examine whether they discriminate between mated and unmated females, whether this discrimination is based on changes in the sexual attractiveness pheromone or the copulatory fluids, and whether they continue to discriminate after the mated females have lost their mating plug. We found that male garter snakes discriminate between the trails of mated and unmated females. This discrimination is based on the presence of a copulatory pheromone, rather than changes in the sexual attractiveness pheromone. The duration of the copulatory pheromone coincides with the duration of the mating plug. Thus, male garter snakes are able to minimize time spent searching for unavailable females by determining female mating status from a distance.

Introduction

In choosing a mate, an individual must evaluate a variety of criteria. A potential mate minimally must be of the appropriate species, sex, and age class. Once these basics are determined, other criteria may be used to determine the quality of one potential mate in comparison to others.

One potentially critical criterion in choosing among potential mates is whether they have recently mated (Wittenberger and Tilson, 1980; Parker *et al.*, 1997). Recently mated females of some species experience a refractory period after mating, during which they are unwilling or incapable of remating. This refractory period may be due to a physiological change (*e.g.* Nilson and Andrén, 1982; Baer *et al.*, 2001) or a physical change such as a mating plug (*e.g.* Devine, 1977; Ross and Crews, 1977, 1978; Orr and Rutowski, 1991; Groot and Smid, 2000; Shine *et al.*, 2000b). Males that continue to court females during the refractory period will suffer the cost of searching and courtship without the benefit of mating. This cost may be manifested as exposure to predators, energetics of locomotion, or missed mating opportunities with other individuals. Thus, it is advantageous for individuals to determine the mating status of potential partners from a distance, thereby minimizing the cost of searching. Males that determine whether females have recently mated will be able to avoid refractory females and direct courtship efforts elsewhere, where they may be more effective.

In some species in which females experience a refractory period, males are able to discriminate between mated and unmated females from a distance. In the mustard white butterfly, *Pieris napi*, females only remate after four to six days. During mating, males transmit an antiaphrodisiac pheromone to females, which makes other courting males quickly abandon them (Andersson *et al.*, 2000). In the orange tip butterfly, *Anthocharis cardamines*, females reject additional courtship after mating by elevating their abdomen. Males court virgin females more intensely than mated females, although it is unclear how they discriminate (Wiklund and Forsberg, 1985). In the Australian swallowtail, *Cressida cressida*, males deposit a large mating plug which is visible from a distance and signals female mating status to subsequent males (Orr and Rutowski, 1991). In the common green capsid, *Lygocoris pabulinus*, a mirid bug, males occlude female reproductive tracts with a mating plug. Male green caspids tested in a Y-maze olfactometer do not prefer the odour of recently mated females to a blank (Groot and Smid, 2000). Examples of male vertebrates determining from a distance whether females are in a post-mating refractory period are limited to the brown lemming, *Lemmus sibiricus = trimucronatus*, and a variety of lizards. In the brown lemming, females become aggressive to conspecifics after mating, and do not accept further courtship. Males tested in a Y-maze olfactometer prefer the odor of unmated to mated females (Huck *et al.*, 1984). In some lizards, changes in throat color indicate when a female is gravid. In most cases, these color changes have been interpreted as aids in sex recognition, reducing male aggression towards gravid females that might otherwise be mistaken as intruding males, and as cues that serve as a visual signal of courtship rejection by gravid females (Cooper and Greenberg, 1992; Watkins, 1997).

In the garter snakes *Thamnophis butleri*, *T. radix*, and *T. sirtalis*, males deposit mating plugs in females that prevent them from remating for two to four days. These recently mated females are courted significantly less than unmated females (Devine, 1977; Ross and Crews, 1977, 1978). However, it is not known whether males can detect recently mated females from a distance.

The red-sided garter snake, *Thamnophis sirtalis parietalis*, is a model species for the study of vertebrate reproductive behavior and pheromonal communication (Mason, 1993). In Canada, individuals of this species hibernate in limestone sinkholes for six to eight months of the year (Gregory, 1977b). These populations mate in the vicinity of these limestone "dens" upon emergence in late April and May. The mating season lasts only six weeks (Gregory, 1974; Shine *et al.*, 2001a), and predation near the dens is high, making the search for mates costly (Aleksiuk, 1977; Gregory, 1977b; Shine *et al.*, 2001b). On average, male snakes emerge before females and remain in the vicinity of the den for seven days, while females emerge over the entire breeding season and leave the vicinity of the den after only one to three days (Gregory, 1974, 1984; Shine *et al.*, 2001a). This pattern of activity results in strongly male-biased sex ratios at the den, where males may outnumber females by more than 15 to one (Gregory, 1984; Shine *et al.*, 2001a).

Skin lipids of female garter snakes contain a sexual attractiveness pheromone and are passively deposited as snakes move over the substrate. These pheromone trails are detected and followed by males in search of females (Ford, 1981; Ford and Low, 1984; Ford and O'Bleness, 1986; Mason, 1992; LeMaster *et al.*, 2001). Upon finding a female, a male competes with an average of three other males (but as many as 100) for the

opportunity to mate (Joy and Crews, 1985; Shine *et al.*, 2001a). After a female mates, her cloaca is blocked by a gelatinous mating plug that physically prevents mating. The area around her cloaca is coated in copulatory fluids which contain the copulatory pheromone (Shine *et al.*, 2000b). Males determine the mating status of females by tongue-flicking in the vicinity of the cloaca and quickly depart if they detect the copulatory pheromone (Devine, 1977; Ross and Crews, 1977, 1978; Shine *et al.*, 2000b). The plug disintegrates approximately two days after mating (Shine *et al.*, 2000b), and females are then likely to remate. Rates of remating in the wild are not known for this population, but in outdoor enclosures 50% of females remate (J. Pasteris and R. T. Mason, unpublished data). In other populations of *T. sirtalis* up to 75% of litters are multiply sired (McCracken *et al.*, 1999).

In this mating system it is important for males to discriminate between mated and unmated females. Courtship of a recently mated female is futile due to the presence of a mating plug. The cost of searching for females can be high, but is reduced by the use of pheromone trails that help males find females more efficiently. However, it is unknown whether males can also use these pheromone trails to discriminate between mated and unmated females, thus avoiding the potentially costly search for unavailable females.

We set out to answer the following three questions concerning male garter snakes' ability to discriminate between the pheromone trails of females. First, can male red-sided garter snakes discriminate between the trails of mated and unmated females? Second, is the ability to discriminate between mated and unmated females based on a pheromone in the copulatory fluids or changes in the sexual attractiveness pheromone? Finally, is this

ability to discriminate between mated and unmated females due to a long-term signal, or does this signal deteriorate after a few days when the mating plug disintegrates?

Methods

We collected 157 male and 80 unmated female garter snakes (*T. sirtalis*) from a hibernaculum near Inwood, Manitoba, Canada (50°31.58'N, 97°29.71'W) on 11 and 12 May 2002, at the peak of the breeding season. Female mating status was determined by examining newly emerged females for a pronounced mating plug. All collected females were reproductively mature (>50cm snout-to-vent length, Shine *et al.*, 2000a). Males and stimulus females were housed separately during the experiment in outdoor 1 x 1 x 1m arenas. Males were allowed to court, but not mate with, females that were not included in this study. Males were housed with females in a sex ratio equivalent to that seen in the field. All animals were released within ten days to the den site from which they were collected.

We tested male trailing preferences using a Y-maze. The maze was constructed of wood and consisted of a base arm (45 x 13.5 cm) and two diverging arms (100 x 13.5 cm) at a 135° angle to the base arm. Each arm had two rows of pegs (6 cm tall) to provide push points for the crawling snake. The rows were 6.5 cm apart, and the pegs within each row were separated by 7.3 cm. The surface of the maze was covered with butcher paper and the pegs were covered with sections of plastic drinking straws. Paper and straws were replaced after each trial to remove any pheromones. The maze was

elevated 90 cm off the floor to discourage subjects from leaving the maze. The maze was built without walls because these snakes are strongly thigmotaxic (Ford, 1986; Costanzo, 1989). Trials were conducted between 0900 and 1600 hours, which coincided with the peak of daily mating activity. Each male was tested only once and then released at the point of capture.

We made trails on the paper by gently holding stimulus female snakes at the neck and just behind the cloaca, and wiping the ventral surface of the stimulus animal up one side of the base arm and across to the opposite arm of the Y-maze. Females were selected at random from the pool of 80 females. Assignment of the trails to the arms was randomized by coin flip. The trails crossed at the junction of the arms so that the subject snake necessarily encountered both trails before proceeding up an arm (Fig. 2.1). The stimulus females were size-matched in all trials involving a choice between two females because males prefer larger females (Shine *et al.*, 2001c) and can judge female size on the basis of skin lipid pheromones alone (LeMaster and Mason, 2002; Shine *et al.*, 2003). Females were size-matched by selecting the female nearest in length and mass to the first, randomly selected female.

We began each trial by randomly selecting a male and placing him into a box (31 x 18 x 9 cm) at the start of the Y-maze. Test males were allowed to leave the box of their own accord through a small hole. Males typically proceeded up the base arm of the maze and then down the left or right arm. Trials were not scored when the male failed to show a pronounced trail contact response (a pause, lowering of the head, and tongue-flicks directed at the substrate, Brown and MacLean, 1983; LeMaster and Mason, 2001). For a trial to be scored, the snake's entire head had to pass a mark 30 cm up either of the arms.
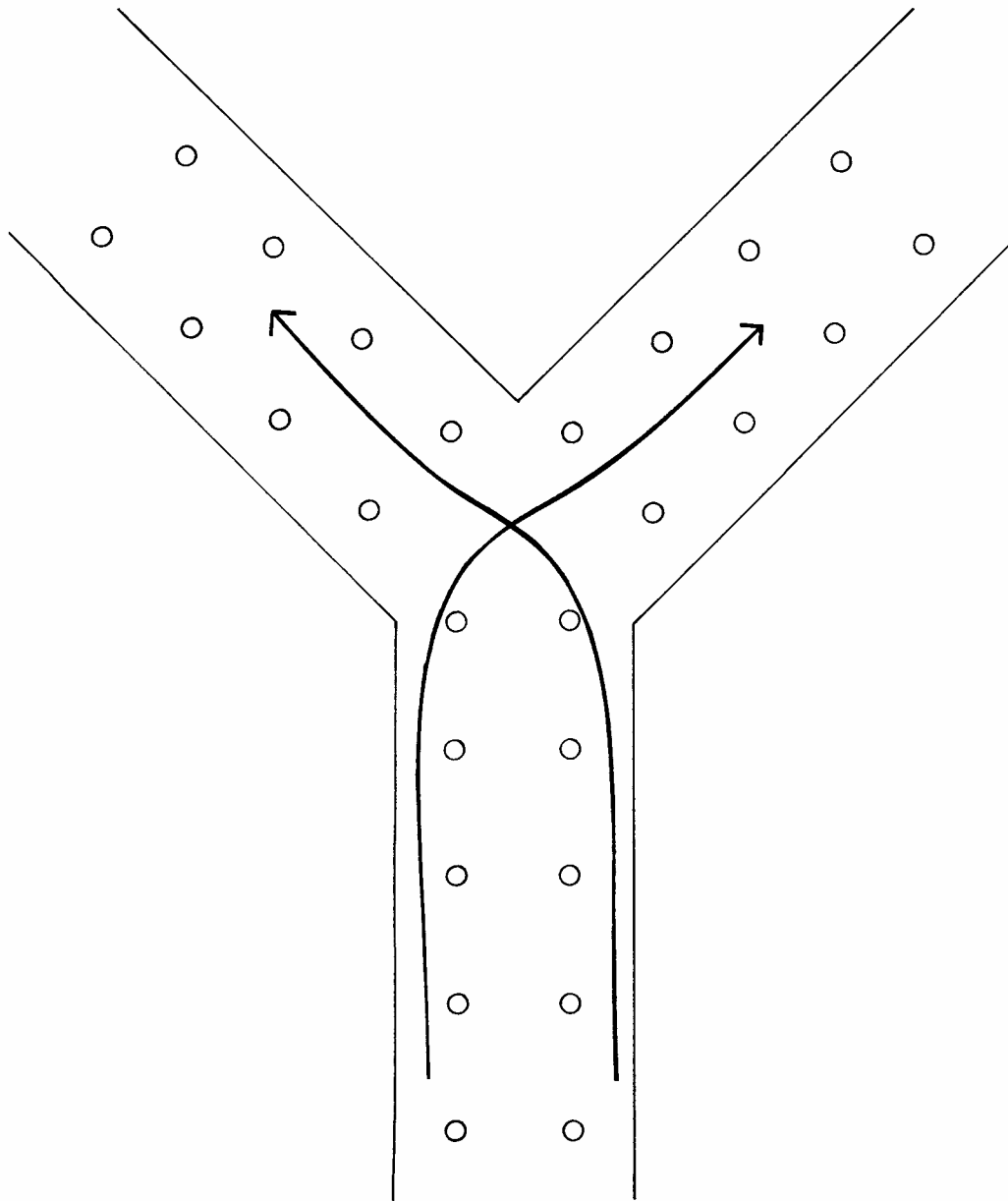
Figure 2.1. Overhead view of the Y-maze. Note that the trails cross at the junction so that the subject male encounters both trails. Modified from LeMaster and Mason 2001.

All decisions on whether to score a trial were made by a second observer who was blind to the treatments. Statistical significance in all experiments was determined with a two-tailed binomial test. Chi-squared tests were used to compare proportions between

selected pairs of trials. All statistical analyses were performed using S-Plus version 6.1 (Insightful Corporation, 2002).

To test for a bias in the Y-maze, ten males were offered the choice between two blank arms. Trail contact responses were not required because no trails were present.

Experiment 1: Can Males Discriminate between the Trails of Unmated and Recently Mated Females?

Our first experiment investigated whether males preferred to follow trails of unmated females to those of recently mated females. We compared male preferences for the trail of an unmated female to a blank arm, a mated female to a blank arm, and an unmated female to a mated female. Mated females had mated less than 60 minutes before the laying of the trail with males that were not used in this study. All female cloacae were occluded with recently deposited mating plugs. Twenty-one unique males were used in each set of comparisons for a total of 63 males.

Experiment 2: Source of the Signal

Our second experiment examined whether the change in female attractiveness after mating was due to the copulatory pheromone in the copulatory fluids or to changes in the chemistry of the sexual attractiveness pheromone in the skin lipids. Males were given a choice between following a trail left by the mid-body of a recently mated female *vs.* one left by her cloacal region. These females had mated less than 60 minutes prior to

the trial. The mid-body trail was laid using the ventral surface of the female from the neck to just anterior to the cloaca. The cloacal region trail was laid using an equivalent length of the ventral surface that included the cloacal region. To control for potential differences in preference between the two regions not due to the copulatory fluids, we also compared male preferences for the mid-body *vs.* cloacal regions of mated females that had lost the mating plug. Twenty-one unique males were used in each set of comparisons for a total of 42 males.

Experiment 3: Duration of the Signal

Our third experiment investigated whether males continued to recognize mated females after the females had lost their mating plug. We compared male preferences for the trails of unmated females to those of mated females that had lost the mating plug and were able to remate. All females that had lost their plug did so spontaneously between two and four days after mating. In addition, we compared male preferences for the trail of a recently mated female to that of a mated female that had lost the mating plug. Recently mated females had mated less than 60 minutes prior to the trial. Twenty-one unique males were used in each set of comparisons for a total of 42 males.

Results

When given the choice between two blank arms, six of 10 males chose the left arm, and four chose the right. There was no preference for either arm of the Y-maze (*P*=0.75).

Experiment 1: Can Males Discriminate between the Trails of Unmated and Recently Mated Females?

Males preferred the trail of unmated females to blank arms and to trails of recently mated females (Fig. 2.2). Almost all males followed the trail of an unmated female over a blank arm (*P*=0.0002). Most males followed the trail of an unmated female over a mated female (*P*=0.007). Although not reaching the level of significance, there was a trend for males to avoid mated females. The majority of males followed the blank arm over the trail of a recently mated female (*P*=0.078). This response was significantly different from their response to the unmated female (χ^2_1=16.70, *P*<0.0001).
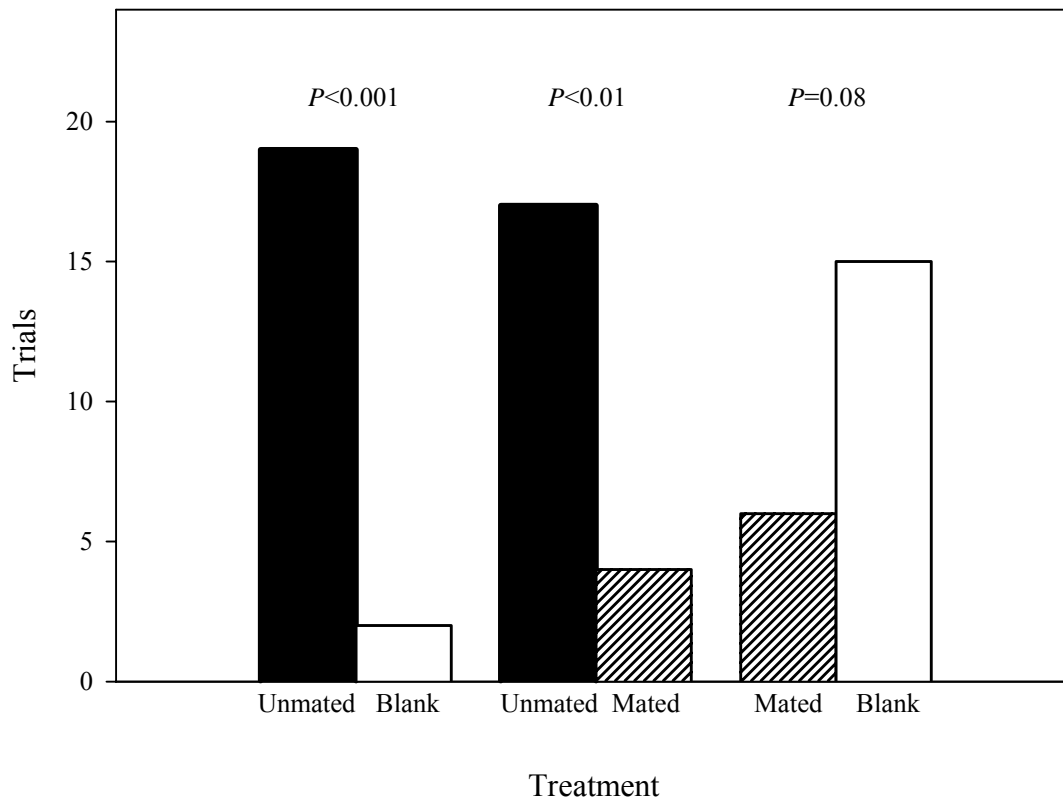
Figure 2.2. Number of trials in which males (n = 21) chose to follow the trail of an unmated female *vs.* a blank arm, an unmated female *vs.* a mated female, and a mated female *vs.* a blank arm.

Experiment 2: Source of the Signal

Most males preferred a trail left by the mid-body of a recently mated female over a trail from the same female's cloacal region (*P*=0.027, Fig. 2.3). This preference for mid-body over cloacal regions was no longer significant after the mating plug had disintegrated. Less than half of the males followed a trail left by the mid body of a mated female that had lost her mating plug over a trail from the same female's cloacal region (*P*=0.38). These proportions were significantly different (χ^2_1=6.22, *P*=0.013).
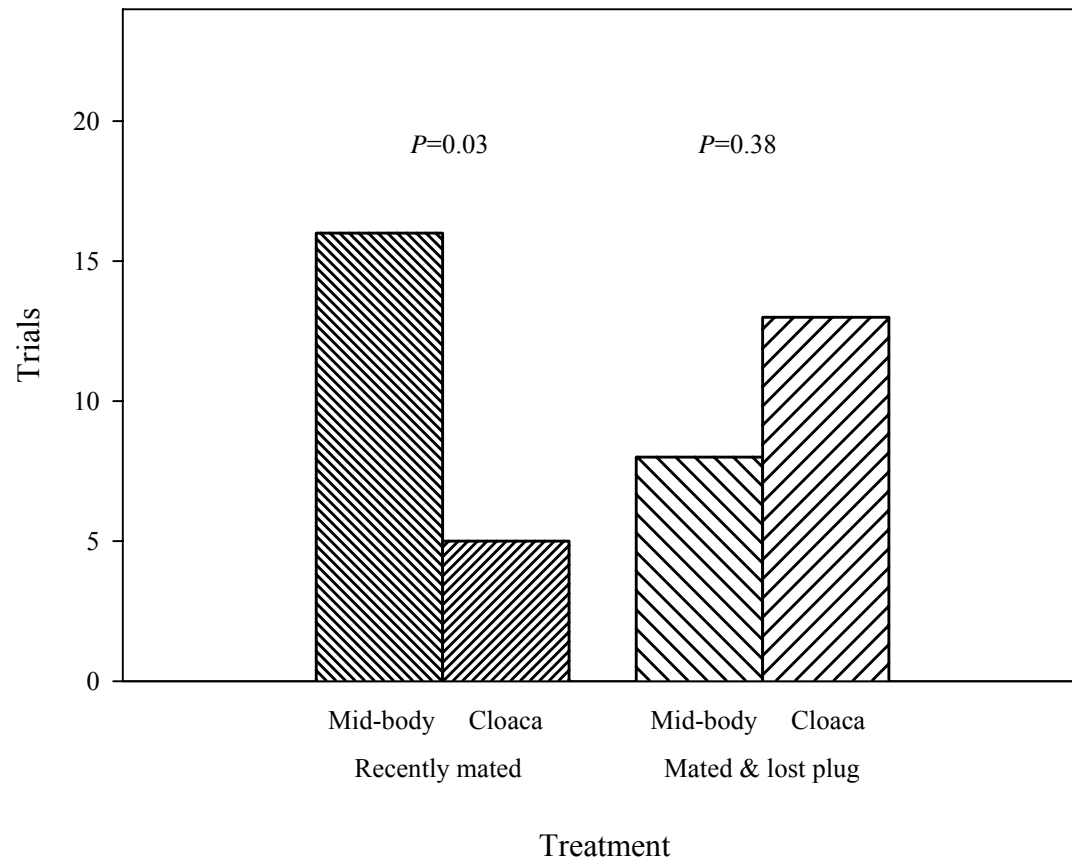
Figure 2.3. Number of trials in which males (n = 21) chose to follow trails from the mid-body *vs.* the cloacal region of a recently mated female, and trails from the mid-body *vs.* cloacal region of a female that had mated but had subsequently lost the copulatory plug.

Experiment 3: Duration of the Signal

There were no preferences for unmated over mated females that had lost their mating plug (Fig. 2.4). About half of the males followed the trail of an unmated female over that of a mated female that had lost her mating plug (*P*=0.66). In addition, males

preferred to trail mated females that had lost their mating plug over recently mated, plugged females. Most males followed the trail of the mated female that had lost her mating plug over a recently mated, plugged female ($P=0.027$). These proportions were significantly different ($\chi^2_1=4.84$, $P=0.027$).
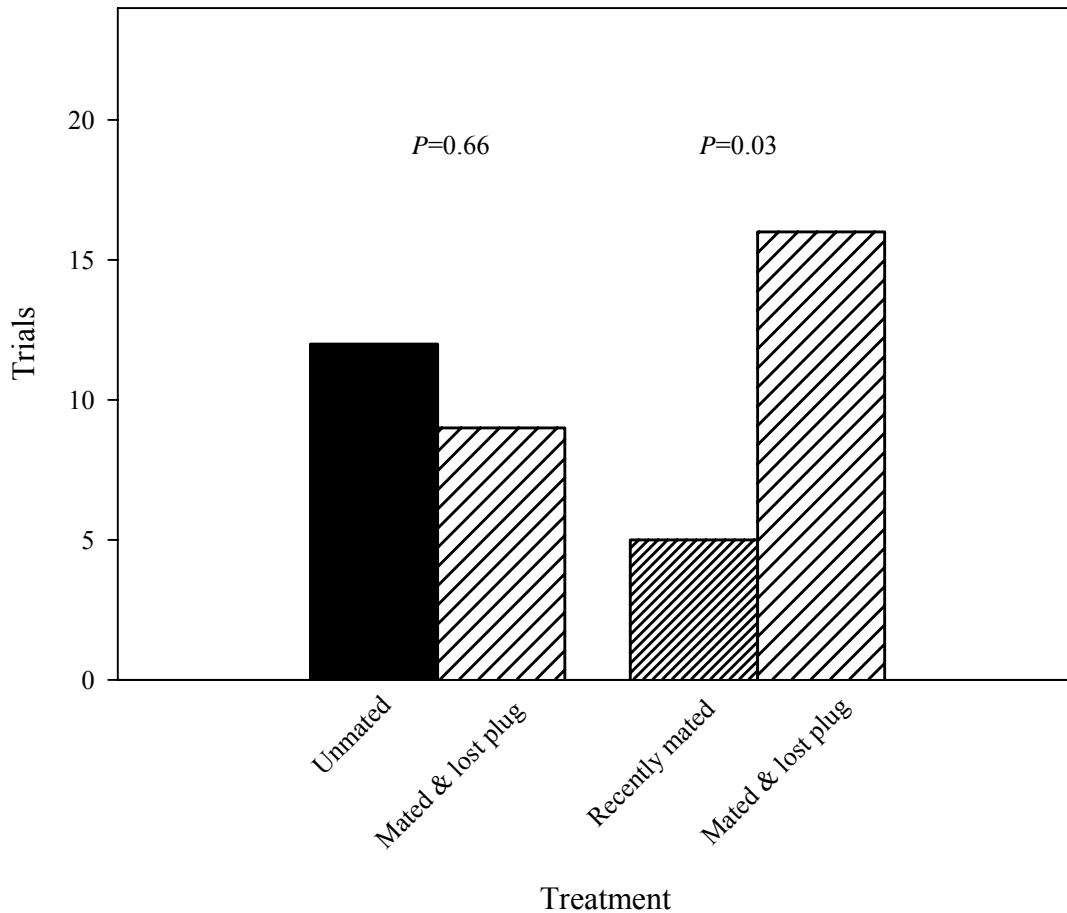
Figure 2.4. Number of trials in which males (n = 21) chose to follow trails from an unmated female *vs.* trails from a female that had mated but had subsequently lost the copulatory plug, and a recently mated female *vs.* a female that had mated but had subsequently lost the copulatory plug.

Discussion

Male garter snakes are known to discriminate between mated and unmated females after they initiate courtship. By tongue-flicking in the vicinity of the cloaca, males can detect the copulatory pheromone and will quickly depart if the female has recently mated (Devine, 1977; Ross and Crews, 1977, 1978; Shine *et al.*, 2000b). However, by the time the male is able to investigate a female's cloaca, he may have already invested considerable time and energy in searching for her. The present research is the first evidence that male garter snakes use pheromone trails to determine female mating status from a distance, and thus avoid a potentially costly search for a mated female.

Our results show that males are able to discriminate between the trails of mated and unmated females. Males follow the trails of unmated females and tend to avoid the trails of recently mated females. The status of a recently mated female is determined via the copulatory pheromone, not changes in the skin lipids. In addition, males no longer discriminate between mated and unmated females after the mated females have lost their mating plug.

Ross and Crews (1977) found that exposure to mated females reduced courtship behavior of males, implying a courtship-inhibiting function of the copulatory pheromone. Devine (1977) reported that unsuccessful male *T. sirtalis* and *T. butleri* in a mating ball quickly disperse, implying a repellent nature of the copulatory pheromone. Interestingly, males in the present study tended to avoid the trails of recently mated females, although not significantly so. However, male responses to the trails of mated and unmated females

were significantly different. We expected that males would follow mated females over a blank arm because there is an 8-9% chance that a plug had not been deposited (Shine *et al.*, 2000b). However, the fact that males tended to avoid the copulatory pheromone may indicate that the pheromone functions as more than an inhibitor of courtship behavior. The copulatory pheromone may actually repel males. Further research is needed to confirm this repellent function of the copulatory pheromone.

The loss in attractiveness of female garter snakes after mating is due at least primarily, if not exclusively, to the copulatory pheromone. Male garter snakes preferred to follow the trail left by the mid-body of a recently mated female over a trail from her cloacal region. This preference is not an artifact of a general preference for mid-body trails because after the mating plug had disintegrated, this preference was no longer seen. In addition, there are no differences between the composition of skin lipids before and after copulation (Mason, unpublished data). These results corroborate the earlier finding that the decline in attractiveness associated with mating is due to a copulatory pheromone contained in the copulatory fluids, and not due to changes in skin lipids (Shine *et al.*, 2000b).

After the mating plug disintegrates, mated female garter snakes regain their attractiveness to a level that is not significantly different from that of unmated females. Males prefer following the trail of mated females that had lost their plug to that of recently mated, plugged females. This preference may simply be due to avoidance of recently mated females. However, it seems more likely that males showed some attraction to females that had lost their plug, because males show no significant preference for unmated females over mated females that had lost their plug. These mated

females that have lost their plugs are available to remate, and will likely do so (McCraken *et al.* 1999; J. Pasteris and R. T. Mason, unpublished data). There is a correlation between the duration of the plug and the duration of the copulatory pheromone. However, this correlation does not imply that the copulatory pheromone is contained in the plug. Rather, the copulatory pheromone is contained in the copulatory fluids (Shine *et al.*, 2000b). The copulatory pheromone indicates recent mating and should only be detected as long as the plug remains effective. If copulatory pheromone levels decline, or the pheromone decays, below the threshold of detection before the plug becomes ineffective, selection would favor males with lower thresholds of detection. Conversely, if the copulatory pheromone lasts longer than the plug, selection would favor males with a higher threshold of detection. The available data support the hypothesis that males detect the copulatory pheromone about as long as the mating plug is effective. For example, after 36-48 hours, 27% of mated females are courted again (Ross and Crews, 1977). During the same period, 21-45% of mated females lose their mating plugs (Shine *et al.*, 2000b). More work is needed to verify this correlation between the duration of the pheromone and the duration of the plug.

Males of some species avoid courting mated females to reduce the risk of sperm competition rather than to avoid a female refractory period (Happ, 1969; Krames and Mastromatteo, 1973). The duration of the copulatory pheromone implies that this is not the case in the red-sided garter snake. If the males were avoiding sperm competition, we would expect them to continue to discriminate against mated females after the mating plug disintegrates. Rather, males do not significantly discriminate against unmated females once the females lose their mating plug. Thus, males are probably not

discriminating against mated females strictly to avoid sperm competition. Males that can mate should do so, even if it means sharing paternity with another male. Only when the female is entirely unable to mate (while she is plugged) should the male not invest in courting her.

It is not clear from this study whether the copulatory pheromone is a product of the male or the female, or both. Ross and Crews (1978) found that vasectomizing male garter snakes before mating blocked production of the copulatory pheromone, but noted that this did not exclude the possibility of contribution by the female. In this population, females benefit from the pheromone because it allows them to avoid further courtship after mating and to escape quickly to the summer feeding grounds, or at least to the aspen groves near the den where the concentration of males is significantly reduced (Shine *et al.*, 2000a; Shine *et al.*, 2001a). Although females will likely mate again, they may be more able to exercise mate choice in these situations. If females produce or contribute to the pheromone, then the decay of the signal after the plug decays is easily explained. Females should not deter suitors from competing for them in the aspen groves. If the males produce the signal, then the decay may be due to a trade-off between volatility of the pheromone (reaching many males) and duration. In addition, it is difficult to explain why mating males would advertise the unavailability of the females with which they mated, given that the mating plug would prevent the females from remating. In the absence of evidence to the contrary, we might predict that mating males would try to draw their competitors toward mated females, distracting them from the remaining available females. Perhaps the copulatory pheromone is not adaptive to the signallers, and instead is an exaptation by the receivers (*sensu* Gould and Vrba, 1982). The second

males may simply be detecting the presence of sperm or seminal fluid left by the mating males. Further research is needed on the source and chemical identity of the copulatory pheromone.

If mating males do produce the copulatory pheromone to lower the chances that a female will remate, then they essentially sequester females with which they mate without having to miss opportunities to mate with other females (Devine, 1984). This sequestering of females is expected to evolve in populations with strongly male-biased sex ratios (Dewsbury, 1982). The lack of a mating plug in other snake species may be explained by the less male-biased sex ratios in those species, where competition for females is less intense. Only eight snake species are known to have mating plugs, and these species tend to mate in large aggregations, although the reproductive behavior of most snake species is not known (Gregory, 1977a; Devine, 1984). A more thorough survey of snake species for the presence of mating plugs would add to our understanding of the evolution of this trait.

Acknowledgements

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CHAPTER III

SEASONAL ANOREXIA IN THE MALE RED-SIDED GARTER SNAKE (*THAMNOPHIS SIRTALIS PARIETALIS*)

Ryan P. O'Donnell, Richard Shine, and Robert T. Mason

Abstract

Many animals show seasonal shifts in behaviors that coincide with breeding, migration, or hibernation. These behavioral shifts provide ideal opportunities to study the regulation of behavior. The red-sided garter snake (*Thamnophis sirtalis parietalis*) spends eight months of the year inactive in underground hibernacula, one month breeding, and three months feeding to build up enough energy stores to survive the following winter. Although they emerge from eight months of hibernation with severely depleted energy reserves, they do not feed until weeks later, after the breeding season. We tested the hypothesis that this lack of feeding during the breeding season is due to a shift in behavior rather than the distribution of food and potential mates. Male garter snakes were given a series of choices between pursuing a breeding or feeding opportunity. The proportion of tests in which males selected feeding over breeding gradually increased throughout the study period, reaching almost 100% in the final tests. Males also were given opportunities to feed and court at the beginning and end of the study. Males initially refused food and courted females, but when retested at the end of the study they fed and did not court females. Thus aphagia during the breeding season is due to at least in part to an endogenous shift in behavior.

Introduction

Many animals experience seasonal shifts in behavior. For example, migration, hibernation, and breeding seasons represent seasonal shifts in locomotion, dormancy, and mating, respectively. Seasonal changes in behavior such as these are extreme, easy to observe, and may provide insight into other, more subtly variable behaviors.

One common shift in behavior is an inverse relationship between breeding and feeding. Not infrequently, both sexes forego feeding entirely during the reproductive season (Mrosovsky and Sherry, 1980; Shine, 1980; Bonnet and Naulleau, 1996). Aphagia (a lack of feeding) during the breeding season may severely deplete energy reserves, and indeed, the ability to maintain high levels of reproductive activity in the absence of feeding has been identified as a major target of sexual selection (Bartholomew, 1970; Andersson, 1994). For example, a dominant male elephant seal (*Mirounga angustirostris*) must remain on his defended beach to maintain control of receptive females throughout the entire mating period before returning to the ocean to feed (Bartholomew, 1970; Deutsch *et al.*, 1990). Breeding male emperor penguins (*Aptenodytes forsteri*) walk up to 120 km to the breeding grounds and remain there without feeding for 115 days (Le Maho, 1977).

The causal basis for such aphagia is complex. At least two different kinds of mechanisms can generate aphagia in reproductive animals: either an intrinsic mechanism (*i.e.*, anorexia, food is ignored even if it present); or an extrinsic

mechanism (*i.e.*, reproduction induces animals to spend their time in habitats where they do not encounter food).

The red-sided garter snake, *Thamnophis sirtalis parietalis*, is an example of an animal with dramatic seasonal shifts in behavior. In the northern part of their range, they are constrained to hibernate underground for eight months of the year (Gregory, 1977b). In late April and early May, they emerge and begin their four-week breeding season. At the conclusion of the breeding season, they migrate to marshes up to 17.7 km away (Gregory and Stewart, 1975). Upon reaching the marshes, they must feed sufficiently in three months to survive the subsequent nine months of aphagia, including another energetically costly breeding season.

When males emerge from hibernation, they immediately begin energetically costly courtship (Shine et al., in press). However, courting males at the dens are never found with food in their stomachs (Aleksiuk and Stewart, 1971; Gregory and Stewart, 1975; Crews *et al.*, 1987). It is unclear whether the lack of stomach contents at the dens reflects an absence of food there, or a refusal to take food even if available (anorexia). Prey items appear to be scarce or absent at the den sites (Aleksiuk and Stewart, 1971; Gregory and Stewart, 1975; Crews *et al.*, 1987). However, males have also been reported to refuse food during the breeding season (Aleksiuk and Gregory, 1974; Crews et al., 1987; but see Shine et al., 2002). During the four weeks after emergence, they eventually stop courting females and migrate to their summer feeding grounds (Gregory and Stewart, 1975; Shine *et al.*, 2001a).

The switch from breeding to feeding in the garter snake offers a robust model system for the study of the regulation of behavior. This system is unique because we

can quantify and compare the extent of an animal's interest in feeding *vs.* reproducing. Snakes of both sexes use chemical trails to locate prey items such as earthworms (Halpern and Martínez-Marcos, 2003), and males use chemical trails to locate potential mates (*e.g.*, Mason *et al.*, 1989; Mason *et al.*, 1990; LeMaster and Mason, 2001). Hence, it is possible to examine and compare the responses of male garter snakes to trails of mating partners and prey items. This system thus allows a direct comparison between a male's interest in mating and feeding, because we can provide each test subject with a choice between the two types of trails and see which he chooses to follow. We can also examine courting and feeding responses more directly, by exposing males to females and to prey and scoring their responses at different times within the breeding season. Finally, this system is ideal because the presence of human observers does not deter the snakes from mating or feeding and simple, unequivocal bioassays are available to quantify these behaviors (Kubie and Halpern, 1975; Halpern *et al.*, 1986; Mason *et al.*, 1989; Mason *et al.*, 1990; LeMaster and Mason, 2002).

Using these bioassays, we sought to characterize the behavioral shift from courtship to feeding, and to determine whether these behaviors are mutually exclusive simply due to their spatial arrangement in the field or to an intrinsic limitation in male behavior. This study takes advantage of the easily quantified behaviors exhibited by a species that not only offers a model for insight into seasonal shifts in behavior, but also the cues that may guide these changes.

Methods

We collected garter snakes from a hibernaculum near Inwood, Manitoba, Canada (50°31.6'N, 97°29.7'W) at the beginning of the breeding season. Twenty males and 40 females were collected on 11 May 2002, and 24 males and 40 females were collected on 9 May 2003. (For the sake of brevity, dates will be referred to by number, with these collection days defined as day 0 within each year.) Snakes were kept at ambient temperatures in nylon outdoor arenas (1 x 1 x 1 m) during the day and in cloth bags at night (LeMaster and Mason, 2002). Each day during the experiment all males were given simultaneous access to three unmated females randomly selected from the pool of 40 to simulate the sex ratio experienced at the den (Gregory, 1984; Shine *et al.*, 2001a). The females' cloacae were taped closed while in the arena so the males could court the females but not mate with them. We performed four experiments to characterize male behaviors in response to cues from prey and females over the course of the breeding season. Experiment 1 was performed in 2002, Experiment 2 in 2003 and Experiments 3 and 4 in both 2002 and 2003. All animals were released at the point of capture upon completion of the study.

Experiment 1: Do Male Preferences for Following the Trail of a Live Female vs. *that of a Worm Change through Time?*

Our first experiment investigated whether male garter snakes' preferences shifted from mating to feeding through time. We gave a group of 20 males the choice between following the trail of an earthworm or that of a female. Although the diet of *Thamnophis sirtalis* varies throughout its range, they commonly eat earthworms, and in some parts of their range worms constitute the majority of their diet (Rossman *et al.*, 1996). Worms are also eaten by *T. sirtalis* at our study site in the Interlake region of Manitoba (Gregory, 1974).

We tested male trailing preferences using a Y-maze. The maze was constructed of wood and consisted of a base arm (45 x 13.5 cm) and two diverging arms (100 x 13.5 cm) at a 135° angle to the base arm. Each arm had two rows of pegs (6 cm tall) to provide push points for the snake. The rows were 6.5 cm apart, and the pegs within each row were separated by 7.3 cm. The surface of the maze was covered with butcher paper and the pegs were covered with sections of plastic drinking straws, all of which were replaced after each trial to remove any chemical cues from the preceding trial. The maze was elevated 90 cm off the floor to discourage subjects from leaving the maze. It was built without walls because garter snakes are strongly thigmotaxic (Ford, 1986; Costanzo, 1989).

We made trails on the paper by holding a randomly chosen stimulus female snake at the neck and just above the cloaca, and wiping its ventral surface up one side of the base arm and across to the opposite arm of the Y-maze. The female was randomly selected from the pool of 40 females, with replacement. To the other arm,

we applied the trail of a worm by dragging a large earthworm along the paper.

Assignment of the trails to the arms was randomized by coin flip. The trails crossed

at the junction of the arms so that the subject snake encountered each trail before

proceeding up an arm (see Fig 2.1).

We began each trial by randomly selecting a male and placing it into an

opaque box (31 x 18 x 9 cm) at the start of the Y-maze. Test males were allowed to

leave the box of their own accord through a small hole. The male typically

proceeded up the base arm of the maze and down the left or right arm. Trials were

not scored when the male failed to show a pronounced trail contact response (Brown

and MacLean, 1983). For a trial to be scored, the snake's entire head had to pass a

mark 30 cm up either of the arms. All decisions on whether to score a trial were

made by a second observer who was blind to the treatments.

Before any trials began, we tested for a bias in the Y-maze by offering ten

males the choice between two blank arms. Trail contact responses were not required

because no trails were present.

Male preferences were tested once every 8-11 days from day 1 to day 48.

For each male, we computed a logistic regression of whether the male followed the

female (0) or the worm (1) as a function of time. A positive slope indicated a trend

from choosing the female to choosing the worm, and a negative slope indicated the

reverse. A slope of zero indicated no net change in preference over time. The slopes

from all males' regressions were analyzed using a two-tailed Wilcoxon signed-rank

test to determine whether they were significantly positive or negative. We also

analyzed the decisions of all males through time in a single, composite logistic

regression, with and without terms for individuals. We compared these regressions with drop-in-deviance tests (analogous to extra-sum-of-squares F-tests, but for generalized linear models such as logistic regression) and assessed the significance of individual terms with Wald's tests (analogous to t-tests).

To determine whether the amount of mass lost affected the change in trail followed, all individuals were weighed to the nearest 0.1 g on day 1 and day 20. The percent of mass lost between these two days was used as an index of mass loss. Mass loss was added as a continuous covariate in the logistic regressions, and the fit was compared with a drop-in-deviance test. All statistical analyses were performed using S-Plus version 6.1 (Insightful Corporation, 2002).

Experiment 2: Do Male Preferences for Following a Trail of Female Pheromones vs. *a Worm Trail Change through Time?*

Our second experiment examined whether a shift in trails followed on the Y-maze through the season (Experiment 1) could be accounted for by shifts in the attractiveness of the females, rather than a change in male priorities. In Experiment 1, female sexual attractiveness pheromones could have changed through time. Any shift in male behavior may have been a result of changes in the stimulus itself, rather than changes in response to the stimulus. Here we used identical stimuli throughout the experiment to control for this possibility. With the exception of the differences noted below, methods were identical to those in Experiment 1.

Twenty-four males were again given a choice between following female and worm trails on a Y-maze. In this experiment, female trails were laid using extracted female sexual attractiveness pheromones (Mason *et al.*, 1990; LeMaster and Mason, 2003). Female extract was prepared by soaking 31 females in 1 liter 100% hexane for 12 h to remove all skin lipids. All females were found recently killed or fatally wounded by crows at the den. Fatally wounded females were euthanized with an overdose of sodium brevital. Care was taken to keep the heads and cloacae out of the hexane to avoid contamination by body fluids. Hexane was then allowed to evaporate until 48 ml of skin lipids in hexane remained. Approximately 0.4 female equivalents of skin lipids in hexane were applied to the Y-maze with a single pass of a fine paintbrush and the hexane was allowed to evaporate from the paper before the trial began. Lipids in hexane extract were stored at -10° C for the duration of the experiment. Male preferences were tested three times, once every ten days from day 4 to day 24. In this experiment, we estimated mass loss using measurements from days 1 and 24.

Experiment 3: Does Feeding Behavior Change through Time?

Our third experiment investigated whether there was a change in males' appetites during the breeding season, without the immediate alternative of courtship. A single large earthworm was placed in a 19 liter container. A male snake was then added to the container, with its head directed at the worm. The snake was allowed

up to 300 s to attack the worm, and latency to attack the worm was recorded. The proportion of snakes attacking the worm was compared between days 4 and 40 in 2002, and days 9 and 24 in 2003. Presence of feeding behavior and latency to attack were compared between dates using a Wilcoxon signed-rank test.

Experiment 4: Does Courtship Behavior Change through Time?

Our fourth experiment investigated whether males' interest in courting females changed during the breeding season, without the immediate alternative of feeding. All males were given simultaneous access to six females. Males were removed from the group when they exhibited courtship behavior, and were given up to two hours to do so. Willingness to court was tested on days 1 and 60 in 2002 and on days 3 and 24 in 2003. Presence of courtship behavior for each male was compared between dates within each year using a Wilcoxon signed-rank test.

Results

Experiment 1: Do Male Preferences for Following the Trail of a Live Female vs. *that of a Worm Change through Time?*

There was no bias in the Y-maze. When there were no chemical cues on either arm, six males went left and four went right (binomial test, P=0.75).

Over time, males switched from following the female trail to following the worm trail (Fig. 3.1). Slopes of individual logistic regressions were significantly positive (two-tailed Wilcoxon signed-rank test, z=3.35, *P*=0.0008).
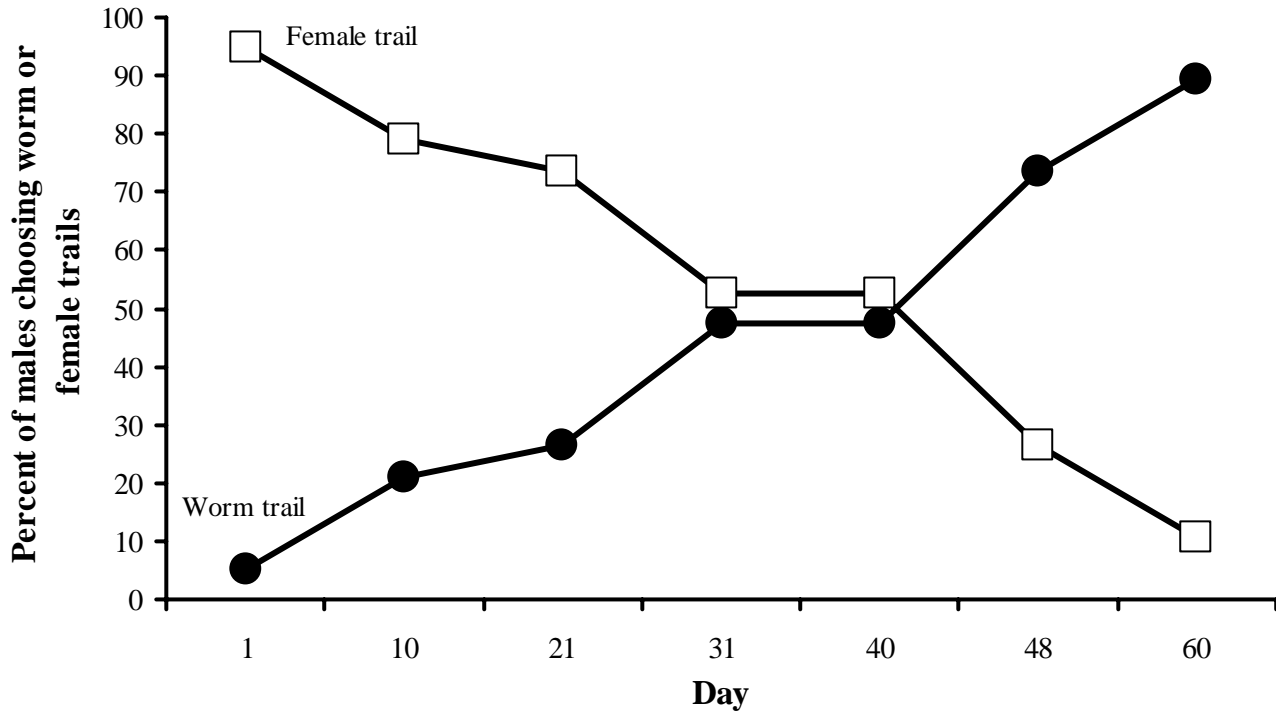
Figure 3.1. Percent of males choosing worm or female trails over time. Data from all males are combined in the figure for clarity.

Time significantly affected the choice when data from all males were combined in a logistic regression (Wald's test, z=4.54, *P*<0.001). However, there were significant differences between individuals in the switch from following one trail to following the other. Allowing for individual difference in slope and intercept

of a logistic regression of choice on time significantly increased the fit over a model

that did not allow for individual differences (drop-in-deviance test, drop in

deviance=84.84, df=38; $P<0.001$). After allowing for individual differences in

response, time still adds significantly to the model (drop-in-deviance test, drop in

deviance=79.85, df=20, $P<0.001$).

Weight loss did not affect the trail chosen by the male. Allowing weight loss,

rather than the individual, to affect the intercept and slope of a logistic regression of

choice on time did not increase the fit over a model that included only time (drop-in-

deviance test, drop in deviance=0.69, df=2, $P>0.25$). Weight loss was not affected

by whether the male ate a worm in the first feeding trial (two-tailed Wilcoxon rank-

sum test, z=-0.48, $P=0.63$).

Experiment 2: Do Male Preferences for Following a Trail of Female Pheromones vs. *a Worm Trail Change through Time?*

There was no bias in the Y-maze. When there were no chemical cues on

either arm, nine males went right, and seven went left (binomial test, $P=0.80$).

Over time, males switched from following the female pheromone trail to

following the worm trail. Slopes of individual logistic regressions were significantly

positive (two-tailed Wilcoxon signed-rank test, z=3.00, $P=0.0027$).

Time significantly affected the choice when all males were included in a

logistic regression (Wald's test, z=2.56, $P=0.01$). Allowing for individual

differences in slope and intercept did not significantly increase the fit when compared to a model that did not consider individual differences (drop-in-deviance test, drop in deviance=60.12, df=46, $P>0.15$). Allowing for an effect of weight loss also did not significantly increase the fit (drop-in-deviance test, drop in deviance=1.51, df=2, $P>0.25$). Weight loss was not affected by whether the male ate a worm in the first feeding trial (two-tailed Wilcoxon rank-sum test, z=0.41, $P=0.68$).

Experiment 3: Does Feeding Behavior Change through Time?

There were significant increases in feeding over time within each year of the study. In 2002, significantly more males fed on day 40 than on day 4 (two-tailed Wilcoxon signed-rank test, z=4.24, $P<0.0001$). Males also attacked the worm faster on day 48 than on day 4 (two-tailed Wilcoxon signed-rank test, z=-4.00, $P=0.0001$). In 2003, significantly more males fed on day 24 than on day 9 (two-tailed Wilcoxon signed-rank test, z=2.33, $P=0.020$). However, males did not attack worms significantly faster or slower on day 24 than on day 9 (two-tailed Wilcoxon signed-rank test, z=-1.71, $P=0.09$).

Experiment 4: Does Courtship Behavior Change through Time?

There were significant decreases in courtship over time within each year of the study. In 2002, significantly more males courted on day 1 than on day 60. Among 20 males, all courted on day 1, and none courted on day 60 (two-tailed Wilcoxon signed-rank test, $z=-4.47$, $P<0.0001$). In 2003, significantly more males courted on day 3 than on day 24. Among 24 males, all courted on day 3, and seven courted on day 24 (two-tailed Wilcoxon signed-rank test, $z=-4.12$, $P<0.0001$).

Discussion

Aphagia in male garter snakes over the four-week courtship period might be caused by a single mechanism. Snakes concentrate on courtship rather than feeding either because of some intrinsic behavioral mechanism (anorexia), or because they do not encounter potential prey in the locations where courtship occurs. Indeed, previous literature has assumed such a dichotomy, leading to directly contradictory conclusions - either that reproducing male red-sided garter snakes refuse to feed even if food is available (Crews *et al.*, 1987; Morris and Crews, 1990) or that the aphagia of these animals is a result of failure to encounter prey rather than their refusal to consume prey if encountered (Shine *et al.*, 2002). Our data resolve this contradiction, showing that both mechanisms may be at work, depending on the

timing of migration from the hibernaculum where courtship occurs to the fields and marshes where feeding occurs. That is, in the early part of the mating period, male garter snakes simply refuse to feed - but at the end of the mating period, they may readily accept prey if it is available. Future work should investigate the coincidence between the timing of this intrinsic shift in behavior and the migration to the feeding grounds.

In years when the intrinsic shift occurs before migration, a single phenomenon (aphagia during breeding) may actually reflect two different proximate mechanisms, both an intrinsic anorexia and an extrinsic absence of prey items at the den. Previous studies had failed to document this complexity, but our study improved upon previous designs by (a) incorporating time within the breeding season as a factor in the analyses; and (b) using a behavioral test (trail-following) that forced males to select between the two competing priorities of feeding *vs.* breeding. This within-season shift in male behavior fits well with other recent research on the Manitoba garter snakes which has revealed substantial shifts in important behaviors over the course of the spring mating period (Shine *et al.*, 2002).

Our experiment using standardized female pheromone trails confirms that the shift in male behavior was dependent on male response to, or perception of, the pheromone. However, this result does not exclude the possibility that the pheromones of the live females changed through the course of the breeding season. Female pheromone profiles do change seasonally, but it is not known whether these changes require a shedding of the skin or on what time scale these changes occur (Mason *et al.*, 1987; Mason, 1992). If changes in female pheromones do occur

during the breeding season, they could partly explain the lowered intensity of courtship to females later in the breeding season (Shine *et al.*, 2002).

While the regulation of the shift from courtship to feeding is not well understood, a number of cues stand out as candidates. One possible cue is weight loss. Males may switch from mating to feeding once they reach a critical reduction in body weight. However, data from this experiment suggest that this is not the case. Weight loss had no effect on the decision to pursue a breeding or feeding opportunity in either year of this study.

The switch from mating to feeding may also be a product of the time required to reactivate the digestive system after eight months of aphagia. However, the shift in *T. s. parietalis* took much longer than required for stomach activation in other snake species. The rattlesnake *Crotalus cerastes* takes 12-24 hours to activate its digestive system after ingesting a meal, and the Burmese python, *Python molurus*, takes 24-48 hours (Secor *et al.*, 1994; Secor *et al.*, 2002). Furthermore, these snakes ingest food while their stomachs are inactive. The presence of nutrients in the digestive tract activates the stomach. It is unlikely that the garter snake delays feeding because the digestive tract is inactive upon emergence because in these other snake species it is the ingestion of food that activates the digestive tract, not vice versa.

Temperature could potentially affect the switch from courting to feeding. The lower thermal threshold for courtship in Manitoba *T. s. parietalis* varies between individuals from 4.5 to 25°C (Aleksiuk and Gregory, 1974; Hawley and Aleksiuk, 1975). Similarly, the minimum temperature required for courtship in Michigan *T. s.*

sirtalis is 4.5°C, and the minimum temperature for copulation is 15.5°C (Blanchard and Blanchard, 1941). Feeding is also affected by temperature. Western terrestrial garter snakes (*Thamnophis elegans*) regurgitate mice if they are ingested when the snake's body temperature is below 10°C (Stevenson *et al.*, 1985). The lower limit for courtship is approximately equivalent to the lower limit for feeding, but there is an apparent upper limit on copulation, if not courtship. Female *T. s. parietalis* with body temperatures over 20°C refuse to mate (Garstka *et al.*, 1982). More data are required on the lower thermal limit of feeding in this population and upper limits on courtship and feeding to determine whether temperature alone could regulate the shift between these behaviors in *T. s. parietalis*. However, both behaviors are observed over a broad range of temperatures. While the chronic thermal regime may regulate the switch, current body temperature alone probably does not.

Changes in day length could potentially regulate the timing of the shift from feeding to mating, as it does in some mammals (Morgan and Mercer, 2001). Because red-sided garter snakes spend 8 months of the year underground in total darkness, light cues are expected to be less important than in other species (Garstka *et al.*, 1982; Whittier *et al.*, 1987). For example, emergence from the hibernaculum, and thus the initiation of the breeding season, is regulated by temperature and not by light (Aleksiuk and Gregory, 1974; Whittier *et al.*, 1987). Captive males switched from breeding to feeding over a period of about 10 days when on an artificial 12L:12D photoperiod after emergence (Aleksiuk and Gregory, 1974), so the switch can occur without any change in photoperiod. The possibility remains that the shift

in behavior may be related to changes in photoperiod; however this hypothesis remains to be tested.

Whatever the environmental cue is that regulates the shift from mating to feeding, the proximate transducer of that signal also remains an enigma. While there could be two separate signals regulating courtship and feeding, the inverse relationship between these behaviors suggests that they may be regulated by the same signal (Aleksiuk and Gregory, 1974). Based primarily on studies in rodents, some specific hormones have been identified as potential regulators of the behavioral switch from mating to feeding (Table 1). These hormones include leptin, neuropeptide Y, galanin, and cholecystokinin, and the steroid hormone glucocorticoids corticosterone and cortisol.

Leptin inhibits feeding and regulates reproduction in mammals (*e.g.*, see Aubert et al., 1998; Auwerx and Staels, 1998), and has been reported to occur in five squamate reptile species (Balk and Niewiarowski, 1998; Secor and Nagy, 2000; Jensen, 2001; Paolucci *et al.*, 2001; Muruzábal *et al.*, 2002). Experimental manipulation of leptin levels in fence lizards, *Sceloporus undulatus*, reduced food intake as it does in mammals (Niewiarowski *et al.*, 2000). When male garter snakes leave the den in late May, their body lipids are at their lowest levels of the year (Aleksiuk and Stewart, 1971) and hence their levels of leptin (produced primarily by adipose tissue) are also likely quite low. This leptin deficiency may cause a decrease in reproductive behaviors and an increase in feeding.

Table 1. Summary of some hormones known to affect feeding and reproductive behavior in rodents

Hormone	Effect on Mating	Effect on Feeding	References
Leptin	required for fertility	decreases	Chehab *et al.*, 1996; Aubert *et al.*, 1998; Auwerx and Staels, 1998; Caprio *et al.*, 2001
Neuropeptide Y	decreases	increases	Clark *et al.*, 1985; Pierroz *et al.*, 1996; Bray, 1997; Aubert *et al.*, 1998; Argiolas, 1999
Galanin	effect varies	increases	Poggioli *et al.*, 1992; Bloch *et al.*, 1996; Crawley, 1995; Bloch *et al.*, 1996
Cholecystokinin	effect varies	decreases	Crawley and Corwin, 1994; Grider, 1994; Argiolas, 1999
Glucocorticoids	decreases	increases	Astheimer *et al.*, 1992

Neuropeptide Y (NPY) increases feeding and decreases mating behaviors in rats (Clark *et al.*, 1985; Pierroz *et al.*, 1996; Bray, 1997; Aubert *et al.*, 1998; Argiolas, 1999). NPY synthesis and release is downregulated by leptin, and NPY is released in response to starvation (Stephens *et al.*, 1995; Aubert *et al.*, 1998; Auwerx and Staels, 1998; Jensen, 2001; Figlewicz, 2003). Both the structure of NPY and the distribution of its receptors are highly conserved among species (Holmgren and Jensen, 2001; Carr *et al.*, 2002), and NPY has been implicated as a regulator of feeding and courtship in *T. s. parietalis*. NPY dramatically reduced courtship and

increased feeding in males (Morris and Crews, 1990). Interestingly, NPY may also be related to aggregative behavior. In *Caenorhabditis elegans*, variation in a NPY receptor homologue affects aggregation (de Bono and Bargmann, 1998). The potential link to red-sided garter snakes, which form the largest aggregations of reptiles in the world, is tantalizing.

Galanin is another peptide that increases feeding behavior, among other functions (Crawley *et al.*, 1990; Crawley, 1995). Like NPY and leptin, it is highly conserved among vertebrates (Jensen, 2001). Galanin may moderate NPY release in rats (Horvath *et al.*, 1996). Although galanin increases feeding, its impact on reproductive behavior has been more controversial. Galanin inhibits reproductive behavior when given intracerebroventricularly in male rats (Poggioli *et al.*, 1992). However, galanin facilitates masculine reproductive behavior when injected into the medial preoptic nucleus of male or female rats in the presence of testosterone, and facilitates feminine reproductive behavior in the presence of estrogen in females (Bloch *et al.*, 1993; Bloch *et al.*, 1996). Galanin's effect on sexual behavior in rats seems to be dependent on the presence of circulating gonadal steroids (Gabriel *et al.*, 1992; Bloch *et al.*, 1993; Bloch *et al.*, 1996). In male *T. sirtalis*, circulating testosterone is high upon emergence from hibernation and rapidly declines during the breeding season (Krohmer *et al.*, 1987; Moore *et al.*, 2000b). Galanin remains a potential regulator of the switch from breeding to feeding in this species, perhaps via its interactions with testosterone and NPY, but this hypothesis remains to be tested.

Cholecystokinin (CCK) is another phylogenetically conserved hormone (Johnsen, 1998) that affects both feeding and reproduction (Argiolas, 1999; Jensen,

2001). While CCK has not been experimentally manipulated in a reptile, CCK has

been found in a turtle, an alligator, and a few snakes, including Burmese pythons,

where plasma concentrations increase 50-fold after ingestion (Jin *et al.*, 1990; Adrian

et al., 1996; Johnsen, 1998; Jensen, 2001). CCK's role in reproduction is less clear.

CCK facilitates lordosis in female rats, but has given conflicting results in males

(Argiolas, 1999). It remains to be seen whether CCK has similar roles in the garter

snake.

The evolutionary advantage of refusing food during the breeding season,

even if food is available, is unclear. The handling time of a worm is not

considerable, taking less than two minutes in most cases (Shine *et al.*, 2002). But

many snakes reduce or cease feeding during the reproductive season (Shine, 1980;

Madsen and Shine, 2000; but see Aldridge *et al.*, 2003). Given the short duration of

the breeding season, the scarcity of prey close to the den, and the very high

emergence rate of females (and thus, the high opportunities for mating), the optimal

male tactic may be to focus on courtship to the exclusion of all else (Mrosovsky and

Sherry, 1980). In keeping with this interpretation, courting male garter snakes show

little overt responses to stress (Moore *et al.*, 2000a) or to predator cues (Shine *et al.*,

2002). Alternatively, refusing food may not be in itself adaptive for snakes. This

seasonal anorexia may simply be due to some physiological constraint inherent with

reproduction. In any case, being willing to ingest food during the breeding season

would be unlikely to present a significant evolutionary advantage in this population

because of the virtual absence of prey items at the dens (Aleksiuk and Stewart, 1971;

Gregory and Stewart, 1975; Crews *et al.*, 1987).

The red-sided garter snake provides an excellent model for the study of certain aspects of the regulation of feeding behavior. While the value of mammalian models of anorexia and obesity with known genetic backgrounds is not to be understated, the dramatic shifts in feeding and reproductive behavior in the garter snake allow the study of the hormonal regulation of these behaviors within a single animal, rather than comparing multiple genetic mutants. In the future, the red-sided garter snake may prove to be a valuable model for the study of the regulation of courtship and feeding behavior.

Acknowledgements

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CHAPTER IV

GENERAL CONCLUSIONS

Summary of Findings

This thesis has explored two ways in which male garter snakes reduce the costs associated with reproduction by adjusting their behavior. I will address the findings of each chapter in turn, and then summarize with a discussion of potential areas for future work.

It has previously been established that males prefer to court females that have not recently mated (Devine, 1977; Ross and Crews, 1977, 1978; Shine *et al.*, 2000b). In Chapter II, we expanded on this earlier finding by showing that males also prefer to follow the pheromone trails of unmated females over those of recently mated females. This preference for unmated females is not due to a change in female skin lipids. Rather, it is due to the copulatory pheromone that is found in the vicinity of the cloaca. We concluded that males do not avoid mated females to avoid sperm competition, but rather are avoiding females that are not capable of copulation due to the presence of a mating plug. Females that have mated but have lost their mating plug are not significantly less attractive than females that have not mated.

By discriminating between unmated and recently mated females from pheromone trails, males are able to determine female mating status from a distance.

It is likely that males use this information in the field to avoid trailing unavailable females. It is critical that they do so because there are high costs associated with searching for females. Among these, trailing males expend energy, are exposed to predators, and miss other breeding opportunities (Gregory, 1974; Aleksiuk, 1977; Gregory, 1977b; Shine *et al.*, 2001a; Shine *et al.*, 2001b).

In Chapter III, we found that male garter snakes show a significant and dramatic shift in behavior over the course of the breeding season. Upon emergence in the spring they court females and show no interest in food. At the conclusion of the breeding season, they feed readily and show no interest in females. A decline in courtship behavior and an increase in feeding behavior contribute independently to this switch. This shift in behaviors is not due to the distribution of mates and food in time and space, but is rather due to an intrinsic shift in behavior.

While the cost of searching for mates is high, as discussed above, it is reduced by behavioral modifications in the breeding season. The search for a mate would be made even more costly if it were confounded by the search for prey. This is especially true in the populations that we studied, where mates and prey are separated by several kilometers (Gregory and Stewart, 1975). A male simultaneously searching for mates and prey would be forced to cover vast distances daily, and would likely be ineffective at both tasks. The same is true in many animals where mates and food are separated spatially and/or temporally (Bartholomew, 1970; Le Maho, 1977; Deutsch *et al.*, 1990; Andersson, 1994).

Our data show that male garter snakes reduce this cost of searching through an intrinsic reduction in appetite (anorexia) during the breeding season. By

inhibiting their appetite, they focus exclusively on mate searching when mates are available. As the females migrate to the feeding grounds and become unreceptive to courtship, male behavior changes and males also migrate to the feeding grounds. This temporal specialization of behaviors is optimal for a situation where the breeding season is short and the locations for feeding and breeding are spatially so far removed.

Opportunities for Future Work

Each chapter presented here offers many possibilities for future work. I have discussed several of these in the discussion sections of the respective chapters. Here I will consider areas of future work that pertain to both chapters as a whole.

Chief among the areas where work is needed on these subjects is in the elaboration of male reproductive costs. As stated in Chapter I, the costs of reproduction in the male have been historically overlooked and underestimated. In fact, costs of reproduction have only rarely been adequately measured in any species (Reznick, 1992). The male garter snake may provide an excellent model system for the quantification of reproductive costs. One great advantage of the garter snake over many other taxa is that there is no paternal care (Shine, 1988). Male investment in reproduction ends at insemination. Thus by studying male investment in courtship and copulation, we can with certainty include all male reproductive costs. In

addition, females also show no parental care (Shine, 1988), so comparisons between genders are facilitated. All female investment occurs between courtship and birth.

Male reproductive costs can be defined narrowly as the energetic cost of courtship and copulation, including mate searching and the production of the mating plug. While some studies have given a preliminary look at the metabolic costs of courtship (Shine *et al.*, in press), these costs deserve further study. Experiments using doubly-labeled water could measure metabolic expenditure during reproduction more directly than the methods that have been employed thus far, which included measurement of lactate levels and weight loss (Mason unpublished, Shine *et al.*, in press).

Using a broader definition of courtship costs, male exposure to predation during courtship should be quantified. While it is clear that males are exposed to high levels of predation during the breeding season (Aleksiuk, 1977; Gregory, 1977b; Shine *et al.*, 2001b), these levels have not been adequately quantified, and no experiments have been done to compare the risks incurred by a courting male versus one that is not courting. Such experiments are needed to better understand the cost of courtship in terms of exposure to predation.

In conclusion, I have analyzed two ways in which male garter snakes use behavior to minimize the costs associated with reproduction. The costs analyzed here are well known qualitatively, but should be better quantified. The actual costs of reproduction remain poorly known both in the male garter snake and in male animals in general. Future work should add to our knowledge of costs of reproduction in both genders, but especially in the historically overlooked male.

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