The Role of Trailing Behavior in Conspecific Mating in *Thamnophis elegans* and *Thamnophis sirtalis*

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THE ROLE OF TRAILING BEHAVIOR IN CONSPECIFIC MATING IN \textit{THAMNOPHIS ELEGANS} AND \textit{THAMNOPHIS SIRTALIS}

by

Eleanor Watson

Capstone submitted in partial fulfillment of the requirements for graduation with

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with a major in

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in the Department of Kinesiology and Health Sciences

UTAH STATE UNIVERSITY
Logan, UT

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Lastly, I would like to thank the Undergraduate Research Fellowship program for getting me involved in research, and all of the learning experiences I have been able to have from participating in it.

Biography

Eleanor is from Birmingham, Alabama and is graduating in Health Education and Promotion. While at USU, she has enjoyed being involved with the Best Buddies club, the Pre-PA club, and undergraduate research. She is planning on applying to physician assistant school and recently accepted a job with University of Utah Health.
The role of trailing behavior in conspecific mating in *Thamnophis elegans* and *Thamnophis sirtalis*

Abstract

In many gartersnake species, successful mating depends on the ability of males to follow pheromone trails left by females. The populations we investigated (*Thamnophis sirtalis* and *Thamnophis elegans*, closely related sympatric species) overwinter together and simultaneously emerge. Although, mating occurs concurrently, there is no evidence of hybridization. Therefore, we sought to investigate the mechanisms that allow male snakes to differentiate between heterospecific and conspecific females to ensure mating success. Behavior studies were conducted by presenting male snakes with extracted scents of conspecific females, heterospecific females, and conspecific males. We measured male preference by number of investigatory tongue flicks and time and percentage of time spent at each scent. Our results support the hypothesis that male snakes prefer the scent of conspecific females, as opposed to heterospecific females. Furthermore, these results suggest that the use of species-specific pheromones is important in distinguishing between closely related species.

Introduction

The ability to reproduce is a hallmark of species success. In order to survive, species must discriminate between conspecifics and heterospecifics when choosing a partner (Lemaster, 2001). In closely related species, this discrimination may be confounded by similarities in mating behavior and close proximity to mating sites (Gartside, 1977). However, this process of identifying and selecting closely related individuals over distantly related individuals can help drive speciation and, over time, separate species even further.

One way of determining conspecific mating partners is the release and reception of chemical messengers like pheromones (Ford, 1986; Mason et al., 1989). Although gartersnakes have generally good visual acuity (Sillman, Govardovskii et al. 1997), olfactory senses play an important role in intraspecific communication in snakes (LeMaster 2001; Madison, 1977).
Epidermal glands likely produce the pheromones used for communication (Madison, 1977). Snakes follow scent trails to locate prey, and male snakes also follow scent trails to find females during breeding season, and form mating balls (Ford, 1986). It is believed that female snakes use pheromones to create scent trails that attract conspecific males. In response to competition, males can mimic female pheromones to enhance their chances of reproductive success (Mason and Crews 1986, Mason 1993). In female snakes, reproductive cycles, including cycles of pheromone production, appear to be regulated by cyclic changes that signal receptivity (Garstka and Crews, 1981). Female snakes create mating trails using a specific pheromone made of homologous chains of saturated and monosaturated methyl ketones (LeMaster and Mason, 2001). Male snakes are attracted to both natural and synthetic products of these saturated and monounsaturated methyl ketones (Mason, Jones et al. 1990). It is produced in the liver of females (Garstka, Camazine et al. 1982), and the estrogen hormone likely controls this production (LeMaster and Mason, 2002). This pheromone was found in skin lipids of mating females during the breeding season, and was not found in skin lipids of males during the breeding season (LeMaster and Mason, 2001).

Several studies indicate that male snakes locate reproductive females by following a pheromonal mating trail that the female leave behind (Ford, 1986; Mason et al., 1989). Aside from olfaction, they may also have the ability to see pheromone with UV sensitive cones (Sillman, Govardovskii et al. 1997). This practice is likely important in populations where closely related species have sympatric distributions. Male snakes should be able to differentiate the species of female by her trail, which raises the possibility that the chemical messengers left by the female serve as species-specific attractants (Ford, 1986).

Snakes tongue flick to detect pheromones in the vomeronasal organ, located on the roof of the snake's mouth (LeMaster, 2001). Therefore, observing snakes' tongue flicks can be used to quantify olfactory activity (Madison, 1977). Previous research found that male snakes could more accurately follow conspecific trails than female snakes (Ford, 1986; Madison, 1977). However, it is not clear whether these same scents are important for species identification. Many snakes of similar species have been known to overwinter together and breed immediately upon emergence (Brown, 1974; Aleksiuk, 1974). In most instances, the observed species are
distantly related, emerge at slightly different times, and/or have different mating strategies (Templeton, 1981). However, in some cases, very closely related species will overwinter together and utilize the same space and time to breed upon emergence. This is the case of two very closely related species, _Thamnophis elegans_ and _Thamnophis sirtalis_ in Cache Valley, UT. In some locations, these two species overwinter and breed at the same location, but there is no evidence of hybridization (Neuman-Lee, pers. obs. Brodie, Jr., pers. obs). While studies have examined how important pheromones are in locating a mate, it is unclear whether these chemical signatures are precise enough to be used as the primary metric for discriminating between similar species. _T. sirtalis_ and _T. elegans_ are closely related. These populations overwinter together and emerge concurrently (LNL, personal observation).

To test the idea that chemical cues are an important signal for species identification in sympatric gartersnakes, the current study exposed males of two different species of gartersnake (_Thamnophis sirtalis_ and _Thamnophis elegans_) to scents collected from female conspecifics, heterospecifics, male conspecifics, and finally a control extract chemical. We then tested the males’ behavioral preference using a behavioral choice experiment, by measuring tongue flicks, absolute time, and proportion of time spent at a given scent. We hypothesized that male snakes would prefer the scent of conspecific females, but would spend less time and tongue-flick less at scents of heterospecific females or conspecific males. Specifically, we predicted that males would preferentially explore the scent of female conspecifics, followed by female heterospecifics, and finally males.

**Methods:**

*Animals and housing*

Snakes were caught by hand at two sites in Cache Valley, Utah in April, 2016 (18 male _T. elegans_, 10 female _T. elegans_, 13 male _T. sirtalis_, and 1 female _T. sirtalis_). Snakes were housed individually in 8-L glass aquaria with newspaper substrate, a water dish and a plastic shelter filled with moist peat moss. Heat tape at one end of the aquarium provided a thermal gradient. The room was kept at 26 °C on a 12:12 on: off light cycle. Snakes were acclimated to laboratory housing for 12 days before the start of experimental trials. All procedures and protocols were
approved by the Utah State University Institutional Animal Care and Use Committee (protocol #2299).

**Scent collection**
Snake scents were collected by moistening a piece of filter paper with Hexane (Fisher H302-4 HPLC grade) and wiping it down the snake’s back twice for each snake (Noble, 1937; Parker, 2011). Following scent collection, the filter paper was added to an additional 20 mL of Hexane per snake and combined to form a solution for males and females of each species respectively. Scents were collected from all female snakes of both species, three male *T. irtalis*, and three male *T. elegans*.

**Behavioral trials**
Behavioral preference trials were run in a 4’ x 4” x 6” glass tank, providing sufficient room for the snake to travel and investigate each end of the tank independently. Three separate tanks were constructed, but only one trial was run at a given time. Prior to the start of each trial, a piece of filter paper was placed at each end of the tank equidistant from the center (Figure 1; i.e., 12 inches from the center of the tank). On one end, 1 mL of snake scent solution was added to the filter paper and on the other end, 1 mL of Hexane was added to the filter paper to serve as a control.

Each snake was exposed to a conspecific female scent, a heterospecific female scent, and a conspecific male scent in three separate trials. Trials for individual snakes were spaced 5-8 days apart to allow the snake to recover. The testing order, scent order, spatial placement of filter paper in the tank (i.e., which tank end the treatment versus control filter paper was placed), and tank used in test (of the three behavioral tanks) were randomized using the Microsoft Excel randomize function.

Each snake was placed in the center of the tank with the head facing forward (not pointed toward either scent) and held for 5 s. Tongue flicks and time were measured when the snake was released. We recorded how many tongue flicks and how many seconds were spent in each section of the tank (center/scent/no scent) until the snake had stopped moving and tongue
flicking for 30 s, was showing no interest in scents, or if it had not moved from the center for 10 min from the beginning of the trial. Behavioral trials lasted until snakes ceased tongue flicking and exploratory behavior (i.e., trials were terminated once snakes had not moved for 10 minutes). Between each trial the tank was cleaned with distilled water and 70% ethanol. Control tests also held with hexane solution + filter paper on both sides of the tank.

Figure 1: Experimental schematic

Statistical Analyses
Analyses were run separately on *T. sirtalis* and *T. elegans* males (Table 1). Tongue flicks per second was log$_{10}$-transformed to meet assumptions of normality and homogeneity. No transformations were successful for percent tongue flicks or percent time, so non-parametric analyses were conducted. The order of the scents was not significant (P > 0.05), so it was removed from the final analyses. Wilcoxon Tests were conducted on the percent tongue flicks and percent time and, when the overall model was significant, comparisons for each pair using pairwise Wilcoxon tests (Table 2). All analyses were run using JMP 12.1.0 (SAS Institute Inc.). Significance level for all tests was set at an $\alpha = 0.05$.

Results
*T. sirtalis*
There was a significant treatment effect for the percent tongue flicks by trial, but not for the percent time by trial or for tongue flicks per second (Table 2; Figure 2). For both percent tongue flicks by trial and percent time by trial, pairwise comparisons revealed a significant difference in *T. sirtalis* males between heterospecific female scent and conspecific female scent, but not between heterospecific female and conspecific male scent, or between conspecific female and conspecific male scent (Table 2; Figure 2a,b).
**T. elegans**

There was a significant treatment effect for both the percent tongue flicks by trial and percent time by trial, but not for tongue flicks per second (Table 2; Figure 2). For the percent tongue flicks by trial, pairwise comparisons revealed a significant difference for *T. elegans* male tongue flicking between heterospecific and conspecific female scents, and conspecific female and male scents, but not between heterospecific female and conspecific male scents (Table 2; Figure 2a). For percent time by trial, pairwise comparisons revealed a significant difference for *T. elegans* males between conspecific male and conspecific female scents, but not for other comparisons (Table 2; Figure 2b).

<table>
<thead>
<tr>
<th></th>
<th>Percent Tongue Flicks @ <em>T. sirtalis</em> (Female)</th>
<th>Percent Tongue Flicks @ <em>T. elegans</em> (Female)</th>
<th>Percent Tongue Flicks @ Male Conspecific</th>
<th>Percent Time @ <em>T. sirtalis</em></th>
<th>Percent Time @ Male Conspecific</th>
<th>Tongue Flicks per second @ <em>T. elegans</em> Males</th>
<th>Tongue Flicks per second @ <em>T. sirtalis</em> Males</th>
<th>Tongue Flicks per second @ Male Conspecific</th>
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<tbody>
<tr>
<td><em>Thamnophis sirtalis</em></td>
<td>0.71 ± 0.07 (n = 11)</td>
<td>0.21 ± 0.09 (n = 10)</td>
<td>0.40 ± 0.12 (n = 12)</td>
<td>0.54 ± 0.09 (n = 11)</td>
<td>0.22 ± 0.09 (n = 12)</td>
<td>0.62 ± 0.09 (n = 11)</td>
<td>0.14 ± 0.06 (n = 12)</td>
<td>0.21 ± 0.06 (n = 12)</td>
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<td></td>
<td>Percent Tongue Flicks by Trial</td>
<td>Percent Time by Trial</td>
<td>Tongue Flicks per Second</td>
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<td></td>
<td>(X^2 = 9.45) (d.f. = 2) (P = 0.009)</td>
<td>(X^2 = 0.07) (d.f. = 2) (P = 0.07)</td>
<td>(F_{(2, 19)} = 2.29) (P = 0.13)</td>
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<tr>
<td>Thamnophis sirtalis</td>
<td>HF CM ((P = 0.19))</td>
<td>CM CF ((P = 0.31))</td>
<td>HF CM ((P = 0.24))</td>
<td></td>
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<tr>
<td></td>
<td>CM CF ((P = 0.09))</td>
<td>HF CF ((P = 0.003))</td>
<td>HF CF ((P = 0.02))</td>
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Table 1. Mean, standard error and sample size for different behavioral trials for different scent types for *T. sirtalis* and *T. elegans.*
<table>
<thead>
<tr>
<th>Thamnophis elegans</th>
<th>( X^2 = 7.13 )</th>
<th>( X^2 = 6.19 )</th>
<th>( F_{(2,31)} = 0.54 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>d.f. = 2</td>
<td>d.f. = 2</td>
<td>P = 0.59</td>
<td>P = 0.03</td>
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<tr>
<td>P = 0.03</td>
<td>P = 0.045</td>
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<tr>
<td>Pairwise Comparisons</td>
<td>HF CM (P = 1.0)</td>
<td>HF CM (P = 0.62)</td>
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<td></td>
<td>CM CF (P = 0.024)</td>
<td>HF CF (P = 0.06)</td>
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<td></td>
<td>HF CF (P = 0.023)</td>
<td>CM CF (P = 0.024)</td>
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**Table 2.** Pairwise comparisons among different behavioral trials for different scent types for *T. sirtalis* and *T. elegans*. Tests were conducted on the percent tongue flicks, percent time, and tongue flicks per second and comparisons for each pair using the Wilcoxon Method.

**Discussion**

Overall, we found support for our hypothesis that male snakes prefer to follow the mating trail of conspecific females compared to females of other species. For both *T. sirtalis* and *T. elegans*, the percent time by trial and percent tongue flicks by trial showed significant preference to conspecific females over heterospecific females. However, our hypothesis that preference of scent would be for females over males, regardless of species, was not supported. *Thamnophis sirtalis* did not show a significant difference in preference between heterospecific female and conspecific male, based on percent time or tongue flicks per second. *T. elegans* did not show a significant difference in preference between heterospecific female scent and conspecific male scent, based on percent tongue flicks or percent time by trial. The results suggest that there are differences in the pheromones produced by females of different species, which are used to attract conspecific males. In addition, male snakes seem to be able to identify species-specific pheromones and follow those mating trails.

Both *T. sirtalis* and *T. elegans* male snakes showed a significant preference toward conspecific female scent. This was demonstrated both by relatively more investigatory tongue flicking and the amount of time spent near conspecific scent (*T. elegans*). These results suggest
that the most critical distinction for male snakes is between conspecific and heterospecific females. This is likely an important distinction because reproductive efforts will only be successful if males are able to find a conspecific female mate. Male snakes are limited by both time and energy when trying to successfully mate.

While conventional wisdom has stated that males emerge first and breed immediately upon female emergence, there is a more nuanced understanding of this relationship. Shine et al. (2001) found that male and female snakes continually emerge, but males remain for longer periods of time at the den site. Mating also occurs near the den site, but also can occur at some distance (Shine, Elphick et al. 2001). Therefore, having the ability to immediately discriminate the conspecific vs. heterospecific scent is important throughout the breeding season. When a female appears, male snakes must compete to breed with the female. Even though gartersnakes have well developed eyes, the visual acuity does not match that of a mammal or bird (Sillman, Govardovskii et al. 1997). This indicates a probable role in olfactory recognition to determine female presence and receptivity. The time frame of mating is a small window during the spring, after which snakes disperse to forage. There is evidence of mating during the fall, prior to overwintering (LNL, unpubl. data), and it is unclear if scent plays a role in this mating at well.

While male reproduction is generally thought to be a low energetic cost, an analysis of sperm plugs in gartersnakes shows that if a male successfully mates with the wrong species, it is a significant energetic waste due to the high energy expenditure in creating sperm plugs (Friesen, Powers et al. 2015). Many male snakes use resources gained from the previous season and are in an anorexic state during spring mating periods (O’Donnell, Shine et al. 2004). Therefore, this energetic investment is not trivial.

The ability to distinguish between conspecific and heterospecific females should be especially important for sympatric species that share hibernacula, like this population of *T. elegans* and *T. sirtalis* (Ford, 1986). Male snakes can identify the species of the female, and if they are sexually receptive from the trail the female has laid. In a trailing behavior experiment, Ford found that sympatric species could distinguish conspecific female scents from other sympatric species. It is likely that sympatric species produce different pheromones in order to create species- specific scent trails (Ford 1986). We found support for this idea, whereby, both species exhibited the ability to differentiate between scents of conspecific females and the scents of other snakes. Snakes of both species showed significant preference toward conspecific
females in measurements of both tongue flicks and time spent exploring scents. However, *T. elegans* showed a stronger preference for the conspecific female scent than was shown by *T. sirtalis* in the behavior trials.

*T. elegans*, but not *T. sirtalis* males, also showed preference toward conspecific female scent over conspecific male scent for time spent and percent tongue flicks. However, we were able to capture more *T. elegans* females and therefore it is possible that the fact that our scent was only from one female *T. sirtalis* contributed to this result. However, there may simply be physiological differences in the production of the scent and/or the sensory system between the two species.

Interestingly, for both species, the average number of tongue flicks per second was not significantly different among any stimuli and thus may not a good metric for preference in this context. Much of our knowledge of gartersnake pheromone communication comes from *T. sirtalis* populations on the northern edge of their geographic range in Manitoba (Seigal, 1996). Our work provides evidence that there are strong chemical cues in *T. sirtalis* from a more central portion of their range as well as the closely related *T. elegans*. This work helps address questions of consistency across home ranges.

A next important step will be to assess mechanistic control of the pheromone release in this system and whether it varies between *Thamnophis* species. Since the endocrine system takes part in regulating reproductive activity and pheromone production (Parker and Mason 2012), it is likely that endocrine control regulates the production and release of species-specific attractant pheromones.

Conclusions
Using pheromones to communicate with conspecific members is common in vertebrates (Lemaster 2001). In snakes as well it is clear that chemical cues are important sensory signals. Snakes follow scent trails to locate prey (Ford, 1986), and male snakes are able to identify the species of female by her trail, which raises the possibility that the chemical messengers left by the female serve as species-specific attractants. (Ford, 1986). The current study adds to this body of knowledge by demonstrating the ability of male snakes to discern between the scents of closely related sympatric species. These results help provide insight into the reproductive biology of reptiles. Over the long term, we anticipate the results will be useful in revealing long
held questions about the role of chemical messengers and mating behavior in mediating reproductive isolation and speciation in general.
Thamnophis sirtalis  Thamnophis elegans

Tongue flicks per second at scent  Proportion of time spent at scent  Proportion of tongue flicks at scent

Conspecific Female  Heterospecific Female

Conspecific Male  Heterospecific Male
**Figure 2.** Male snakes' behavioral responses to conspecific female scent, heterospecific female scent, and conspecific male scent as measured by a) proportion of tongue flicks, b) proportion of time spent, and c) tongue flicks per second. Asterisks denote statistically significant differences ($\alpha = 0.05$).

References


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Eleanor Watson

Capstone Reflection

The end product for my capstone project is a written research article of my research on garter snake trailing behavior and how it contributes to conspecific mating. I hope to publish this project in a herpetology journal. I have been working on this research project for about two years from the beginning of the process. Overall, this capstone project helped learn about the research, experimental, writing, and publication process.

I have been writing up my research, editing drafts, and hope to submit it soon for publication. I met with my mentor every Monday to go over the draft and make edits for the next week. I also have submitted drafts to other peers and mentors for review.

Writing this capstone project has been one of the final stages in the research process for the research project I have been working on for the last few years. I have had to do a lot of research, especially in order to write introduction and methods section. I compiled all the data and results of the information I have gathered and collected in my experiments. I wrote results and discussions for what conclusions may be drawn based on the results of my research and experiment.

My mentor was especially helpful during this step of the research process. I had no idea how to write a paper like this. She met with me every week and helped me break down the process into more manageable steps. She gave helpful feedback and input. I have also been especially appreciative of the other members of the lab who have been willing to read and edit my paper. I am sure their experience and aid will also be very helpful to me in the future for the final steps of submitting the paper for publication.
What I have found to be most challenging about this capstone project is that it is my first time planning and carrying out my own research project, and it takes a lot of practice to learn how to go about it. I have written research papers before, so that knowledge and practice has been helpful. However, this paper is much more in depth and advanced than anything I have written before. It has also been an adjustment to write a scientific article as it would be in a research journal.

This capstone project has been challenging and rewarding, because this research project is putting together everything I have learned in my undergraduate research experience. It is the final product of my own research project I have been working on for over two years.

This experience has gotten me more interested in pursuing research in my future career in medicine. I have enjoyed this research experience and find it most rewarding to be able to share what I have learned with other people who can benefit from it. After participating in this research, I think I would be interested to do medical research later in my career. I hope to continue seeking out research opportunities to get myself to that point.

The next step in this process will be to continue getting feedback and edits from the other authors of the paper. Next I will incorporate any of their changes. After that I will choose which journals I want to try to get my paper published in, format the paper to fit the specific requirements for that journal, and submit it for publication. I am interested am excited to see how these next few steps turn out.

I think that it will be very rewarding if my research gets published. It will be cool to see how my work and efforts have paid off, and it will be cool to see that I am able to share the knowledge I have gained with a wide audience of people who are in the related field.
One of the reasons why I have enjoyed participating in research and doing this capstone project is because I want to share my findings with other people. I am applying the knowledge I learned from my research process, and the results of my experience, and sharing it with other people in the field of biology, as well as other students.

I think that this capstone project could be helpful to those who are interested in learning more about how snakes, or reptiles in general reproduce. I learned useful information about the methods these animals use in order to find suitable mates. I also am including in the paper research that will be helpful to people wondering how different species living in close proximity interact with each other, specifically in the mating process.

I have really enjoyed my experience doing research and participating in the Honors Program at Utah State. It has been rewarding to complete this capstone project to see my experiences and knowledge be applied.

My advice to future students starting their capstone would be to stay organized and manage your time well. I would also recommend that they get involved in research that is interesting to them, but still challenging. I enjoyed getting out of my comfort zone and usual academic experiences during this research experience. I also would recommend to find a mentor that you work well with, and people you enjoy working with. I have found that these relationships are very beneficial not only through the research process, but for future job and career opportunities, and education. I think that forming good relationships with the people I worked with will be a lasting benefit of doing undergraduate research.