Determining Male Choice: Pheromone use in Timber Rattlesnakes (*Crotalus horridus*)

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The focus of my proposal is to develop a greater understanding of snakes', specifically the timber rattlesnake's, use of pheromone trails. I would like to address the question of choice. The factors that play into males choosing the "most fit" female are unknown for pit vipers. Deciding to spend the energy to follow a female must be triggered by something in the pheromone trail. This has been studied is certain species of snakes, such as the garter snake, but never studied to the extent to which I am proposing. Also, male-male combat is known to be energetically expensive, but it is still known to occur. Determining when to fight versus when to avoid another male before meeting face to face would save energy that could be allocated towards finding a female or hunting. Male snake choice has not been studied in regards to pheromones and conspecific trailing at all.

Pheromones, a type of chemical cue used amongst reptiles, have been hypothesized to be linked to many more behaviors than anticipated. These behaviors range from repellent, alarm behaviors to sexual behaviors (Mason and Parker, 2010). Many behaviors associated with chemical cues have been studied, but in very few focal species such as lizards and snakes (Mason and Parker, 2010). Some species have been studied greatly while for other species, research is barely scratching the surface (Mason and Parker, 2010). For example, crocodilians have been found to actively secrete a pheromone from a specific gland, but they are unsure for what reason (Mason and Parker, 2010). However, a specific pheromone, the sex attractiveness pheromone, has been identified in garter snakes (Mason and Parker, 2010).

In garter snakes, the pheromone allows males to asses the body length and condition of potential mates where larger, fatter females are more attractive than smaller, skinner ones (Shine et al., 2003). They are hypothesized to be one of the main ways male snakes are able to locate and determine a reproductively available, or gravid, female (Mason and Parker, 2010). Males have been seen trailing other males in the European whip snake, but the reason is unknown (Fornasiero et al., 2007). However, it is unknown if pheromone trails are also produced by males. If so, it is possible that they communicate their fighting ability to other males. Male-male combat is typically a ritualized behavior used by males in order to gain access to possible mates (Jellen et al., 2007). Male-male combat is known to be expensive and therefore difficult to study in the field because of it (Jellen et al., 2007). It is unknown to research what mechanisms are used to avoid a fight or determine if it is worth having, but typically, heavier males are found to mate with females, either by winning a fight or by another reason unknown (Jellen et al., 2007).

Timber rattlesnakes (*Crotalus horridus*) have also been documented to partake in male-male combat (Brown and MacLean, 1983). They are also one species that has been found to use pheromone trails left by conspecifics to find one another as neonates (Brown and MacLean, 1983). Since this species demonstrates both these behaviors, I would like to test the following hypotheses: First, males trail females for mate locating purposes and male timber rattlesnakes trail conspecifics and differentiate between female and male conspecifics. Second, males differentiate between "more and less fit" conspecifics, both female and male. Finally, when given the option between a "more or less fit" female with the presence of another male, male timber rattlesnakes will avoid the other male at all cost.

Animal Collection. During the beginning of the first breeding season, I will collect 40 males (8-11 years old) (Aldridge and Brown, 1995) and 40 females (ages 9-12 years old) from the Adirondack Forest Preserve at 10 different hibernation dens with 4 males and 4 females collected from each (Brown, 1991). Snout-to-vent length and body mass will be measured for each animal with the goal of getting a large range of body condition (Greene, et al., 2001). Collection will occur in mid to late May, before most females enter ovulation, to ensure the females have not mated, then housed until they become gravid (based on Brown, 1991 methods), approximately late May or early June (Martin, 1993). Hopefully, all females will be gravid.

Y-maze Design. A standard Y-maze will be used where the focal male is located at bottom of the Y-maze and is given the option to travel down a fork of the Y-maze (Greene et al., 2001). The structure of the Y-maze will be the same as Ford, 1982, and Brown and MacLean, 1983, expect 3 times the size. Paper will be placed at the bottom of the maze and removed after every trail. The maze will be cleaned thoroughly in between each trail (Greene et al., 2001).

Sex Differentiation Experiment Design. To support the hypothesis that male timber rattlesnakes trail conspecifics and are able to differentiated between males and females, multiple combinations of Y-maze tests with a focal male must be run. This study will start immediately after females are determined to be gravid. To characterize this ability (1) neither sex trail will be placed on either side of the Y-maze, but controls will be placed (N = 40 trials), (2) a gravid female versus a control (N = 40 trials), (3) a male trail will replace the female in experiment one (N = 39 trials), and (4) one male and one female trail will be placed on either side of the fork (N = 39 trials) (Greene et al., 2001). In experiment 2, since it is breeding season, it is assumed that males will follow the female in order to elicit copulation (Greene, et al., 2001). However, since this has not been studied in the timber rattlesnake, 10 males will be allowed to follow 10 gravid females down the Y-maze into a housing box in order to see if courtship and copulation will occur.

All trails will be overlapped before the division of the fork and applied immediately before the focal male is released into Y-maze (Greene et al., 2001; Ford,1982). Males will only be used once throughout these tests and not against their own trail (Greene et al., 2001). In order to acquire the skin lipid trails, a randomly selected snake of the required sex will be encouraged to travel down a piece of paper which will be placed on one fork of the Y-maze (Ford, 1982). If the animal does not travel down this piece of paper, the animal will be housed in a separate area with a paper base for one hour before the experiment in order to collect skin lipids (Ford, 1982). The focal animal will be placed into a box that is connected to the Y-maze and allowed to acclimate for 5 minutes before the trailed paper is placed into a randomly chosen arm (Brown and MacLean, 1983). Once placed, the focal male will be allowed to enter the Y-maze through a door on the side of the box and is determined to be “trailing” once his head has entered the final box at the end of a fork (Greene et al., 2001). If the focal male displays any defensive behaviors or does not display behaviors that are of those correlated to pheromone trailing, the trail will not continue or count towards the final statistical results (Brown and MacLean, 1983).

Sex Differentiation Prediction. If, in experiment 1, the focal male favors one side significantly, there must be an investigation into the set-up of the Y-maze and why the snakes prefer this one side. However, if there is no significant side, then experiments can continue. It is predicted that the male will follow the female in experiment 2, the control path over the male path in experiment 3, and the path of the female over the male in experiment 4. If the focal male follows the path of the female over the control, then it can be concluded that males can trail females. If the focal male follows the control path over the male conspecific path consistently, then the focal male is avoiding the possibility of male-male combat as it is expensive (Jellen et al., 2007). However, if it is not consistent, then it is possible that there are other factors that dictate male-male conspecific trailing. If the focal male follows the female in experiment 4, then it can be determined that the focal male is able to differential between male and female conspecifics.

More vs. Less Fit. To test the second hypothesis, at the end of these experiments, the snakes must be categorized as “more or less fit” based on body condition (Aubret et al., 2002). Body condition will be calculated in the same way as in Aubret et al. (Aubret et al., 2002). “More fit” is defined as high-body condition (HBC), above average, for both males and females (Aubret et al., 2002). “Less fit” is defined as low-body condition (LBC), below average, for both males and females (Aubret et al., 2002). The 5 most and 5 least fit males, and 5 most and 5 least fit females will be humanely euthanized following the procedures in Mason et al. at the end of the breeding season (Mason et al., 1989). These individuals will then be immersed in hexane for 12hr to collect skin lipids and GCMS analysis will follow the procedures conducted by Parker and Mason (Park and Mason, 2011).

GCMS Predictions*.* During the GCMS analysis, all possible differences in skin lipids will be analyzed and correlated to body condition. If a difference is seen in skin lipid composition or amount relative to body condition and/or sex, then this is one mechanism used by snakes to differentiate fitness and/or sex. If this is also true in timber rattlesnakes, then long-chain methyl ketones, similar to those of garter snakes, would be found in the skin lipids of females and lesser to nonexistent in males. If there is a difference between male and female skin lipid compositions or amounts, then this is the mechanism by which snakes use to differentiate between the sexes. If there is a difference between HBC and LBC females, then this could be the mechanism by which a male snake is able to differentiate and, as a possible result, choose a HBC female over a LBC female. However, if this is not the case, then most likely males use a different mechanism to choose between females, if they choose at all. If there is a difference in between HBC and LBC male skin lipid composition or amount, then this is a possible mechanism which male snakes choose to undergo combat with another male.

Fitness Differentiation Experiment Design. During the next breeding season, to test the possibility of male choice the same Y-maze and methods will be used. The snakes not used in the GCMS study, but used in the sex differential study (*N* = 20 of each sex) will fed different diets throughout the non-breeding season in order to get fatter HBC females/males and skinnier LBC females/males. All females used will be gravid and unmated. I will use (1) an HBC female vs. a LBC female (*N* = 20 trials), (2) two females of equal body condition, HBC vs. HBC/LBC vs. LBC (*N* = 20 trials), (3) an HBC male vs. a LBC male (*N* = 20 trials), (4) two males of equal body condition, HBC vs. HBC/LBC vs LBC (*N* = 20 trials). Assuming that the focal male differentiates between high and low body condition male and female conspecifics, I would test the following comparisons: (5) a LBC female versus HBC female with males of equal, low, and high body conditions following the HBC female (*N* = 30 trials, 10 trails/male body condition), (6) a LBC versus HBC female with a male of equal, low, and high body conditions following the LBC female (*N* = 30 trials), (7) a LBC female versus HBC female with males of equal or high body conditions following the HBC female and males of equal or low body conditions following the LBC female, and (8) a LBC female versus HBC female with males of equal or high body conditions following the LBC female and then males of equal or low body conditions following the HBC. All body conditions are relative to the focal male's body condition (low and high).

Fitness Differentiation Predictions *(Test 1-4).* Most likely the focal male will follow the HBC, more fit female in experiment one, assuming there is a difference between skin lipid composition or amount (Aubret et al., 2002). If this occurs even without the difference in skin lipids between high and low body conditions, then another mechanism is being used and laid down during motion by the males and females (Aubret et al., 2002). If there is an HBC female that is consistently preferred over another then there is something else that makes one female more attractive than another that is not being tested. If LBC females are being preferred over high-body condition females, then body condition is not what has the male choose that female. If the focal male chooses to follow a LBC male over a HBC male, then it can be assumed that the focal male is doing so to avoid injury or death. *(Tests 5-8).* Since male-male combat is expensive, I predict that males will avoid other males completely, choosing to follow LBC when another male of any body condition follows the HBC female. If this is does not occur, then it is possible that other factors besides body condition play a role in determining whether or not to pursue male-male combat. If another male of any body condition follows the LBC female, the focal male will follow the HBC female. However, if a male is present on both LBC and HBC female pathways, then the focal male will follow the LBC male or the male of equal body condition, always avoiding the HBC male. It is also possible that the focal male will not travel down any pathway containing another male's pheromone trail.

Significance. This research will aid conservationists and those who create species survival plans to better understand the reproductive systems of the timber rattlesnake. We have seen this in other species, such as the brown tree snake (Greene et al., 2001), the European whip snake (Fornasiero et al., 2007), and the garter snake (Parker and Mason, 2011; LeMaster and Mason, 2001; Shine et al., 2003) to different extents. However, there is a lot of speculation into how snakes choose a mate over large, expansive areas. Choice is being addressed here for the first time in snakes as well. This study also plays a part into better understanding the pheromones used by reptiles.

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Animal Care Questionnaire:

A. Does the proposed study involve endangered species or threatened populations? (Yes/No)
    a. No
B. Does the proposed study involve trapping, netting, banding, or in any other way be potentially disturbing to wild populations? (Yes/No) *If you selected Yes, please elaborate on your relevant experience. (Required)
    a. Yes: I am not experienced on capturing wild rattlesnakes, however I anticipate working alongside someone who I such as Gordon Schuett. I do know how to handle snakes as I have worked at a zoo before.
C. Does the proposed study involve the maintenance of animals in captivity? (Yes/No)
    a. Yes
D. Describe the facilities and the animal care protocols. Be concise, but complete.
    a. Care will follow the procedures of the AZA Animal Care Manual for the Eastern Massasauga Rattlesnake (Sistrurus catenatus catenatus) as it is the only care manual written for rattlesnakes and these snakes have similar habitats. https://www.speakcdn.com/assets/2332/eastern_massasauga_rattlesnake_care_manual_2013.pdf
E. Will the animals be subjected to pain? (Yes/No) *If you selected Yes, have alternative procedures been considered? Please elaborate your answer, including the procedures to be used to minimize pain.
    a. No
F. Will animals be killed? (Yes/No) *If you selected Yes, what methods of euthanasia will be employed?
    a. Yes - by procedures described in Mason et al., 1989