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Location of Pheromone Production and / or Expression in Red-sided Garter Snakes (Thamnophis sirtalis parietalis)

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Location of Pheromone Production and / or Expression in Red-sided Garter Snakes (T*hamnophis sirtalis parietalis)*

By
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An Honors Thesis Submitted in Partial Fulfillment of the Requirements for Graduation from the Western Oregon University Honors Program

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Abstract:

Pheromones play a central role in chemical communication. Previous research has examined pheromone production in many insect species, but little is known about pheromone production in vertebrates. Previous pheromone research has occurred using the red-sided garter snake, *Thamnophis sirtalis parietalis*, as a model system but it remains unclear where the female sexual attractiveness pheromone, the primary pheromone utilized by this species, is being produced. Snake skin epidermis cells have shown to be important in the production of skin lipids and regulating the permeability of the skin, and thus could play a central role in pheromone production and / or expression. This study measured the thickness of the top three epidermal layers in the skin of female red-sided garter snakes throughout the hibernation period. Of the three layers analyzed, the beta keratin layer was the only layer found to show a significant difference in thickness across the sampling periods. For this layer, I observed a decrease in thickness throughout hibernation, supporting a potential role for this layer in pheromone expression in the epidermis for this species.

Acknowledgements:

First and foremost, I would like to thank my thesis advisor, Dr. Michael LeMaster. Without his guidance and suggestions throughout the entire project I would never have finished. I would also like to thank my parents who provided emotional and financial support throughout my undergraduate career. They were available every day to help me keep moving forward, when all things seemed futile. Last but not least, I would like to thank the WOU Honor's Program and Dr. Gavin Keulks for forcing me to grow and learn through this project.

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Introduction:

The ability to communicate effectively is vital to the survival of an animal. There are several signaling methods by which animals communicate, but regardless of the method, the functions for communication are relatively common. For example, animals may want to warn each other of imminent danger; this can be observed when the white-tailed deer raise their tail and snort to warn others of a predator (Hirth and McCullough, 1977). In addition, animals will use signals to mark territories, which is commonly seen where male roe deer scrape or rub their antlers on trees around the environment to mark and maintain their territory (Johansson et al., 1995). Finally, communication between animals is also critical when they want to reproduce. Birds often use song and dance to attract mates during the breeding season. For example, collared flycatcher males will sing and display to females with the females watching and listening to these signals and choosing mates based on the best performances (Eriksson and Lars, 1986).

The types of signals used by animals for communication vary depending on the life history of the species. Some bird species, such as blackbirds, use both visual and auditory means to communicate. If either the coloring of the feathers or the ability to produce sound is dampened, then the ability to control a territory is lowered resulting in lower reproductive success (Peek, 1972). Frogs mainly rely on sound to communicate during the mating seasons. For many anuran species, such as the bullfrog, the calls must be extremely precise before a partner will respond to a call (Capranica, 1966). Another form of communication observed in nature is based on tactile cues. Chimpanzees display this type of communication where touch is

used to gain another's attention (Leavens et al., 2009). Although it is often overlooked, particularly in vertebrate species, chemical communication is also a reliable means of transferring information among animals.

Chemical communication is characterized by the release into the environment of specific chemicals which are received by another animal and cause changes in the physiology and / or actions of the receiver (Dawkins, 1995). These chemical cues are called semiochemicals, for which there are several recognized types. First, kairomones are signals that are produced by one species that when detected by another species, result in harm to the sender (Alcock, 1982). This is seen when predators of bark beetles eavesdrop on the chemical signal emitted by a bark beetle, following the signal to the tree where the bark beetle is nested (Borden, 1985). A second form of semiochemical is termed an allomone which is a chemical signal sent out to the benefit of the sender and results in harm for the receiver (Alcock, 1982). Bolas spiders use allomones to draw in male moths by releasing a chemical that mimics the female moth mating signal (Stowe, 1988).

The last type of recognized semiochemicals are pheromones. Pheromones are chemical cues produced by one individual that affects the behavior and / or physiology of another member of the same species (Karlson and Luscher, 1959). Pheromones are known to regulate multiple behaviors. First, pheromones can mediate aggregation behavior such as that observed in California spiny lobsters that form large groups during the day to provide a higher level of protection from predators (Zimmer-Faust et al., 1985). Second, some pheromones function as alarm signals, which warn animals of immediate danger. Aphids release a pheromone after

being attacked, which warns other aphids nearby that a predator is near (Hardie and Minks, 1999). Third, many insects use cuticular pheromones to distinguish the relationships between the animals around them. Honeybees use this type of pheromone to differentiate between those born to the hive and those that are not; in fact, they can distinguish to the point of recognizing half-siblings separate from full siblings (Breed, 1998).

A last category of pheromones are reproductive pheromones. These signals are used to either attract or dissuade mates and are commonly observed regulating insect reproduction. The North American tiger moth offers a classic example of how pheromones influence mate choice. Male tiger moths gather alkaloids from a plant diet and store them in various areas during development including the integument and cocoon. Adult male tiger moths produce a measurable pheromone signal, hydroxydanaidal, based on the amount of stored pyrrolizidine alkaloids still present during adulthood in the integument; larger males will have stored more alkaloids and therefore derived higher pheromone levels. Males then transfer alkaloids and other nutrients in a spermatophore to the female during mating which provides the alkaloids needed for defense of the offspring until they are able to collect it themselves. The males with the largest alkaloid stores are chosen more often than those with smaller stores due to this "honest" signaling because a greater pheromone level correlates with greater defense capabilities in offspring and therefore increased chance of offspring survival (Eisner and Meinwald, 1995).

While much is known about reproductive pheromones in insects, relatively little is understood with respect to vertebrates. Among fish, female goldfish release

pheromones during reproduction to stimulate oocyte maturation and increase sperm production in males (Koboyashi et al., 2002). Fish also use reproductive pheromones to attract mates to their location, as observed in lamprey (Stacey and Sorenson, 1999). Within amphibians some male salamanders take a different approach by directly injecting the pheromone into the female's blood system using specialized teeth resulting in a female more readily accepting the male's spermatophore (Houck and Reagan, 1990). Alternatively, other terrestrial salamanders transfer similar functioning pheromones from male to female by the contact of skin (Rollmann et al., 1999). For mammals, male elephants release strong smelling "musth" that attracts female mates (Rasmussenet al., 2002) while many mice species choose mates based on the odor of urine (Kavaliers and Colwell, 1995).

One of the best vertebrate models for pheromone research has been the red-sided garter snake, *Thamnophis sirtalis parietalis*. This species of snake is found throughout the mid-western United States and up into Canada. At their northernmost limit, these snakes hibernate in limestone crevices to escape the harsh Canadian winters (Garstka et al., 1982). When spring arrives, male and female red-sided garter snakes start to emerge from the crevices. These aggregations of snakes exiting winter hibernacula can reach levels in the tens of thousands of snakes. When the female emerges from the hibernation den she is quickly surrounded by a ball of up to 100 males each competing for the chance to mate (Mason, 1993).

What attracts the male red-sided garter snakes to the female is a pheromone found on the dorsal surface of the snake (Noble, 1937). This pheromone was

identified as a combination of 18 long-chain saturated and monounsaturated methyl ketones (Mason et al., 1990). These chemical cues are nonvolatile and are categorized as a contact pheromone, evidenced by the required tongue-flick touch of the male on the dorsum of the female before the male will initiate courtship behavior. Following spring emergence, the pheromone expressed in the skin lipids of the female changes its composition to a less attractive profile within weeks after the female emerges (Uhrig et al., 2012).

While it is known that the pheromone is expressed in the skin lipids of the female, it is not yet known where exactly in the skin the methyl ketones are produced. The purpose of my research is to histologically examine the skin of the female garter snake to gain early insights into where the pheromone may be produced.

The outer epidermis layer of a snake is composed of three cell layers (Figure 1). The top two layers are comprised of dead cells containing two types of keratin, a protein similar to what composes the hair and nails of humans (Marieb and Hoehn, 2016). The thick top layer of cells contains beta keratin which helps keep shape and form in the snake scales (Lillywhite, 2014). The layer of cells right underneath the beta keratin layer is comprised of cells containing alpha keratin, which does not have a prominent function other than separating adjoining layers. The deepest third layer is the stratum germinativum, which contains living cells that will divide and differentiate to later become new alpha and beta keratin layers.

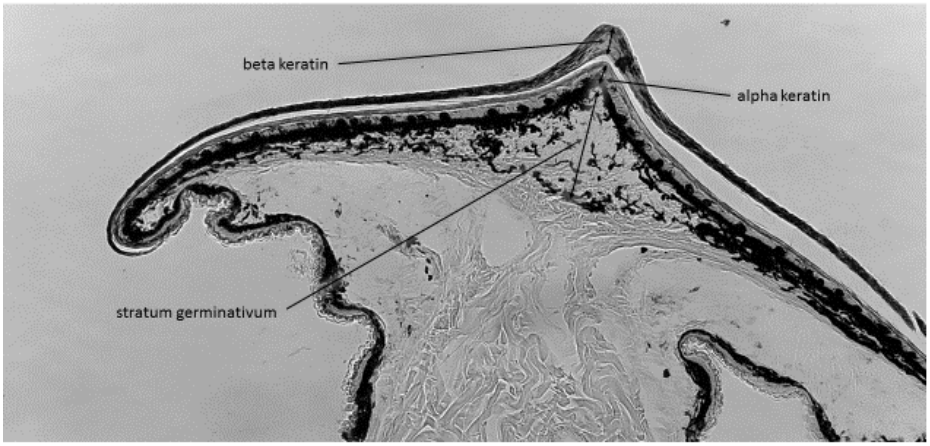

Figure 1: Histological image of the epidermis of the red-sided garter snake. Three layers can be observed: the outer beta keratin layer, the middle alpha keratin layer, and the innermost stratum germinativum.

Recent work has determined that the expression of the female sexual attractiveness pheromone in red-sided garter snakes occurs about mid-way through winter while the females are still hibernating (Parker and Mason, 2009). Utilizing groups of female snakes sacrificed at various times before, during, and after hibernation, I made histological slides of the skin from the dorsum of the female and measured the thickness of the beta keratin, alpha keratin, and the germinatival layers to see if differences in thickness existed across the hibernation period for the three layers. If there is a significant increase in the thickness of a particular layer or layers in the integument during hibernation that correspond with the timing of pheromone expression, then this could indicate that the epidermal layer or layers are the site of pheromone production or, if layers are reducing in size, could allow for the pheromone to more easily reach the surface of the snake.

Materials and Methods

The experimental protocols used in this experiment were approved by the Portland State University Animal Care and Use Committee and were in compliance with the guidelines established by the National Institute of Health Guide for the Care and Use of Laboratory Animals. This research was performed under the authority of Wildlife Scientific Permit WB12691, issued by the Manitoba Department of Conservation. Animals sacrificed for this project were part of larger projects at Portland State University and additional tissue samples were collected at the time of sacrifice which are not discussed in this volume of work.

Animal Collection

Female red-sided garter snakes (*Thamnophis sirtalis parietalis*) were collected from a single den site in the Interlake region of Manitoba, Canada during 1) the fall pre-hibernation period when snakes return to their den site in preparation for winter dormancy (September 2012), and 2) the spring post-hibernation period when snakes emerge to begin the breeding season (May 2013). A total of 50 adult, female snakes were collected: 40 females during the fall pre-hibernation period and 10 females during the spring post-hibernation period.

Animals were weighed upon capture and their snout-vent length (SVL) was measured. To examine variation in skin morphology before and after hibernation, a subset of females collected during the fall pre-hibernation (n = 10) and all of the females collected during the spring post-hibernation period (n = 10) were euthanized immediately after capture with a lethal overdose of 1% sodium pentobarbital (0.2 ml / snake). A small skin sample was then collected from the

dorsum of the snake near the mid-section of the body. To examine variation in the skin morphology during the hibernation period, the remaining 30 snakes were transported back to Portland State University where they were immediately placed in 4°C walk-in coolers to mimic a natural hibernation period. Within each cooler, snakes were housed in glass aquaria with water provided ad libitum and the lights remained off for the duration of hibernation. At 4 weeks, 8 weeks, and 16 weeks following the onset of hibernation, a subset of females (n = 10 for each time period) was randomly selected and euthanized and a small skin sample was again collected from the dorsum near the mid-section of the body.

Skin Tissue Preparation

Skin samples collected from female snakes were immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and then transferred to 0.1 M phosphate buffer and stored a 20C until sectioning. Skin samples were cryoprotected in 30% sucrose in 0.1 M phosphate buffer and cut on a cryostat (Leica 3050S) into 4 alternate series of 25-µm sections (10 sections / slide). Tissues were thaw-mounted on subbed slides (Fisherbrand Superfrost Plus) and stored at -20C until staining.

Slide Staining and Imaging

To visualize the various epidermal layers, a standard haematoxylin and eosin (H & E) staining protocol was followed. Briefly, slides were first placed in a phosphate buffer solution (PBS; 5 min.) and then placed in a solution of PBS and Triton 100x (5 min.), making the cell membranes permeable so that stain could enter the cells. Slides were then placed in haematoxylin stain (1 min.), rinsed in

nanopure water (15 dips), placed in 0.3% acid alcohol (3 sec.), rinsed again in nanopure water (2 dips), and finally placed in a mixture of eosin stain with alcohol (1 min.). Slides were dehydrated through a series of alcohol washes (70%, 80%, 95%, 99%, 100%, 100%; 10 sec. per wash) and washed in Citrisolv (1min.). Coverslips were then over-layed on the slides using Permount to seal the two layers together.

Stained tissue was examined using a Leica DM500 microscope with a Leica ICC50 HD camera and Leica LAS EZ 3.0.0 software. For each tissue sample examined, a single skin scale was randomly selected and images were taken (40x magnification) for all sections of that particular skin scale on the slide (n = 10). For each image, a bar scale (100 μm) was superimposed and the thickness of the beta–keratin, alpha-keratin, and stratum granulosum layers were determined using ImageJ software (National Institute of Health; Bethesda, Maryland). Once thicknesses were calculated, an overall average was calculated for each layer from each individual snake using measurements from the ten replicate snake scale images.

Statistical Analysis:

Statistical analyses were performed using Jandel SigmaStat software (version 12.5, Systat Software, Inc.) One-way ANOVA was used to compare the thickness of each skin layer region among the treatment groups. If significant differences were observed, Tukey's post hoc tests were used to make pairwise comparisons. In addition, regression analysis was performed to determine if there was a correlation between time period sampled and thickness of skin layer for each of the skin layer regions observed.

Results:

A total of forty five skin samples were successfully analyzed across the five treatment groups (Fall = 10 samples; 4-week hibernation = 9 samples; 8-week hibernation = 9 samples; 16-week hibernation = 9 samples; Spring = 8 samples). We observed a statistically significant difference in the thickness of the beta keratin layer across all treatments (ANOVA; F= 2.651; P= 0.049; Fig. 2). Pairwise comparisons (Tukey's tests) indicated that the beta keratin layer was significantly thinner for female's following spring emergence compared to 4 weeks into hibernation ($P<0.05$). In addition, we found a significant negative correlation across the treatments (regression analysis; F = 7.661; P= 0.008; Fig. 3) demonstrating that the beta keratin layer progressively decreased in thickness across the hibernation period.

With respect to the alpha keratin layer, we did not observe a significant difference in thickness (ANOVA on ranks; H= 3.074; P=0.546; Fig. 4) or a correlation between layer thickness and time period sampled (regression analysis; F = 0.005; P = 0.994; Fig. 5). The same was true for the stratum germinativum where there was neither a significant difference in layer thickness across sampling periods (ANOVA; F= 1.048; P= 0.395; Fig. 6) nor a correlation between layer thickness and time period sampled (regression analysis; F = 2.848; P = 0.099; Fig. 7).

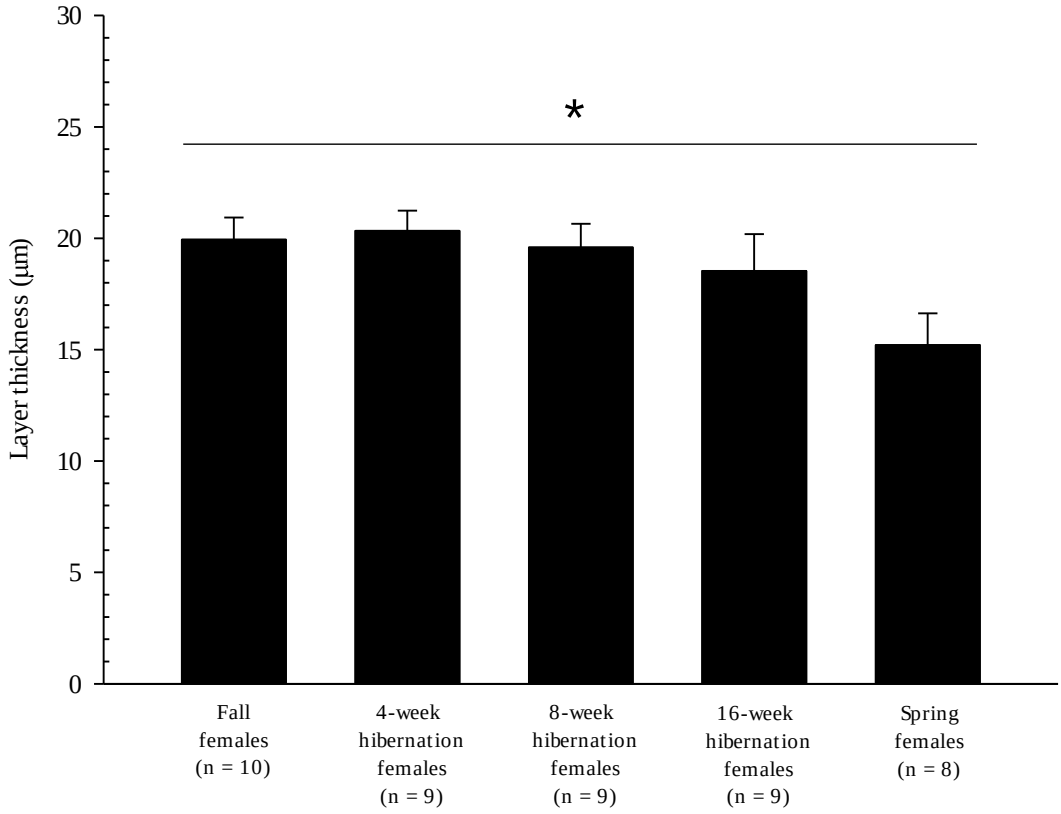

Figure 2: Comparison of beta-keratin epidermal layer thickness across five sampling periods for female red-sided garter snakes. Samples were taken immediately before hibernation, during hibernation, and immediately following spring emergence.

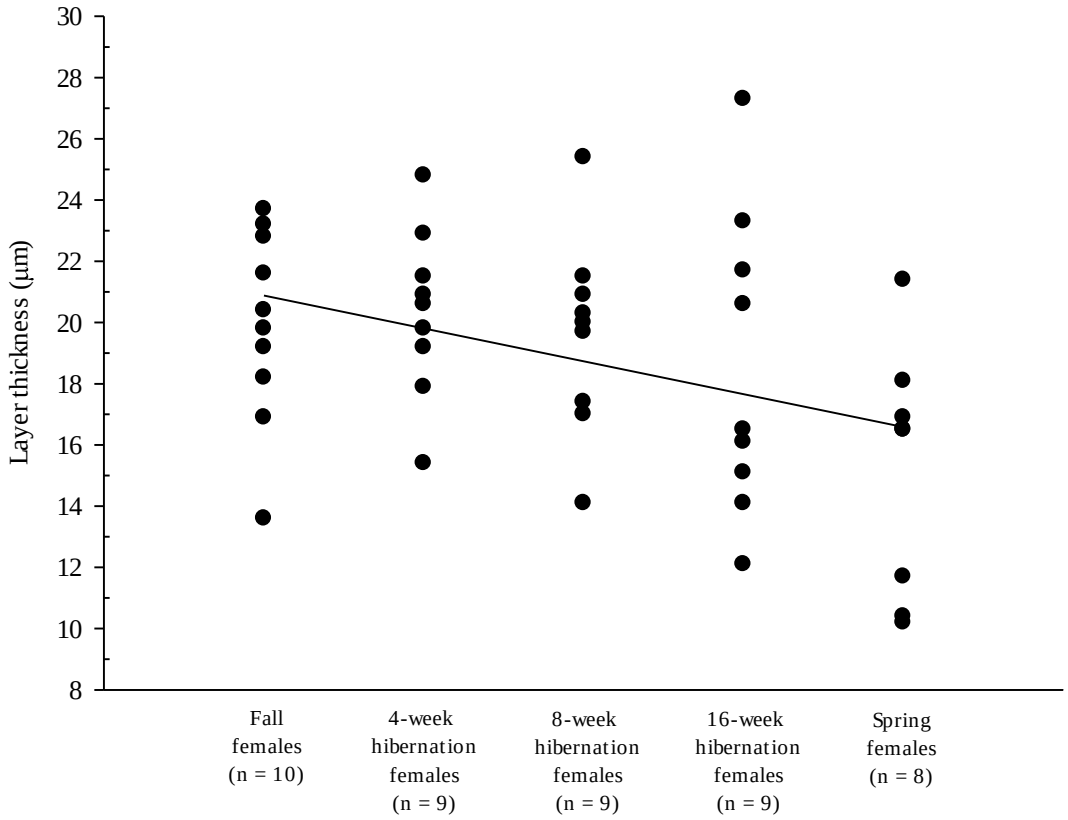

Figure 3: Regression of beta layer thickness versus sampling period for female red-sided garter snakes. Samples were collected at five time points before, during, and following hibernation.

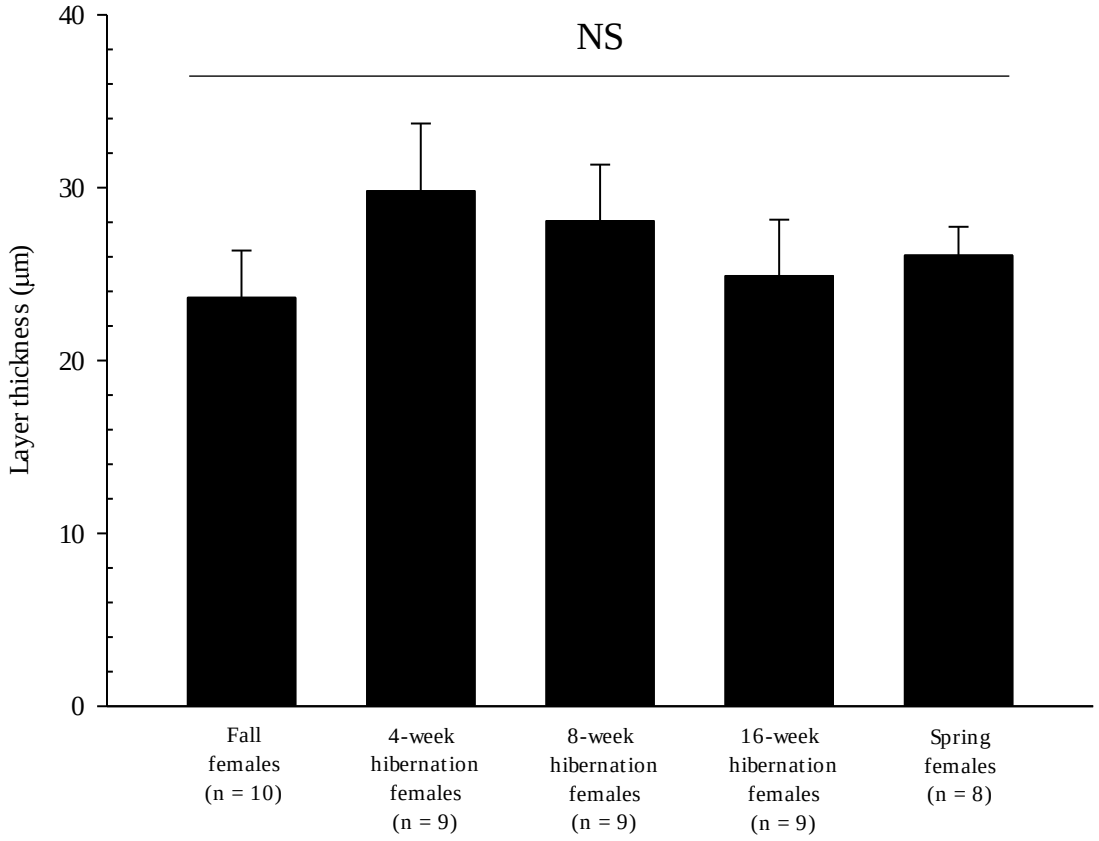

Figure 4: Comparison of alpha-keratin epidermal layer thickness across five sampling periods for female red-sided garter snakes. Samples were taken immediately before hibernation, during hibernation, and immediately following spring emergence.

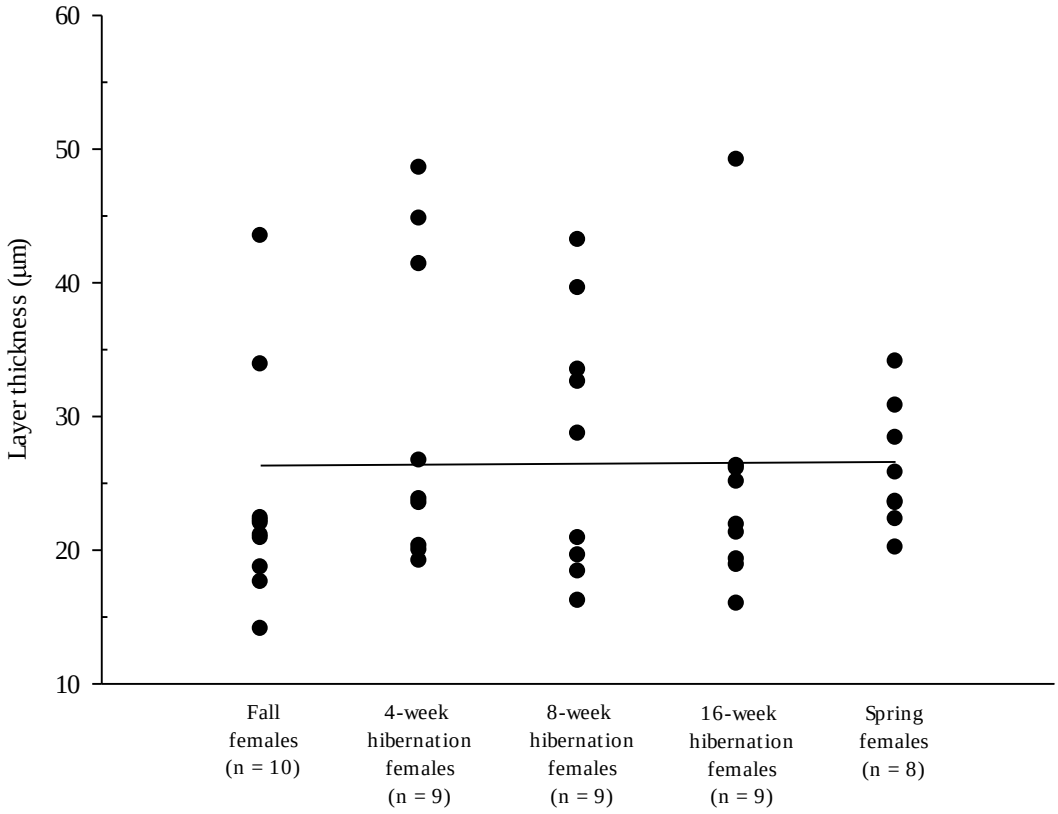

Figure 5: Regression of alpha layer thickness versus sampling period for female red-sided garter snakes. Samples were collected at five time points before, during, and following hibernation.

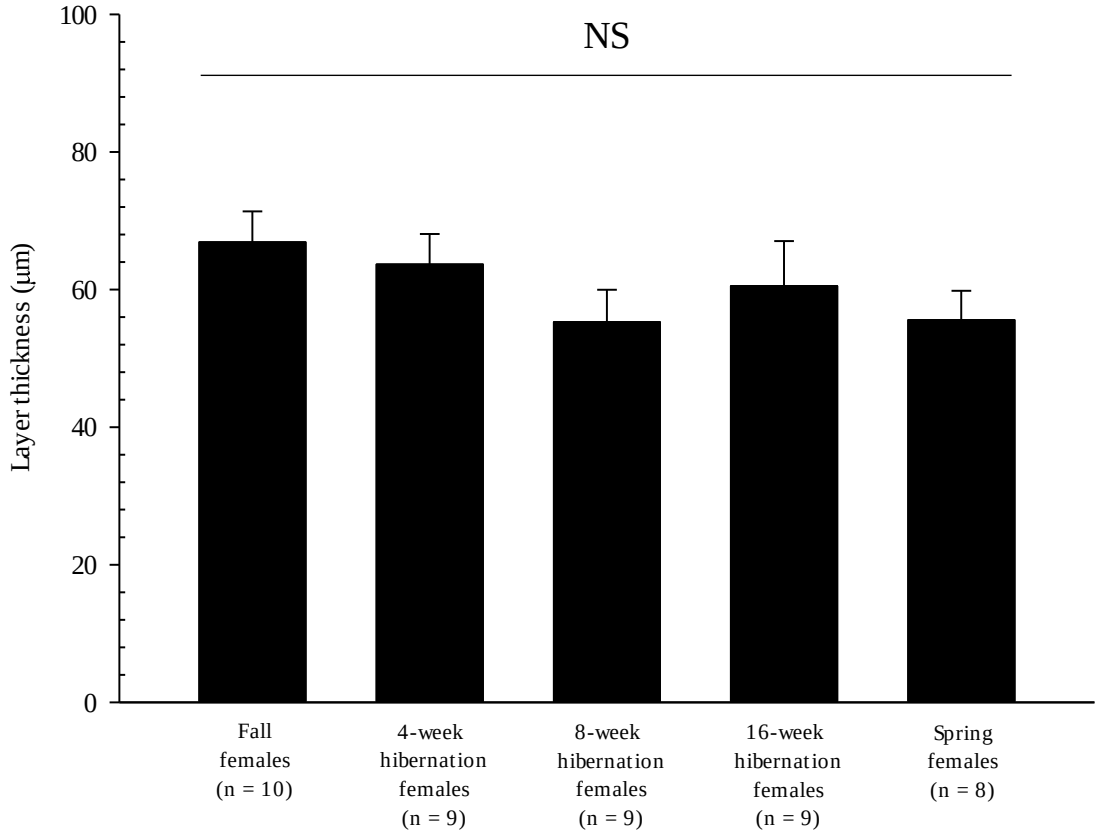

Figure 6: Comparison of stratum germinativum epidermal layer thickness across five sampling periods for female red-sided garter snakes. Samples were taken immediately before hibernation, during hibernation, and immediately following spring emergence.

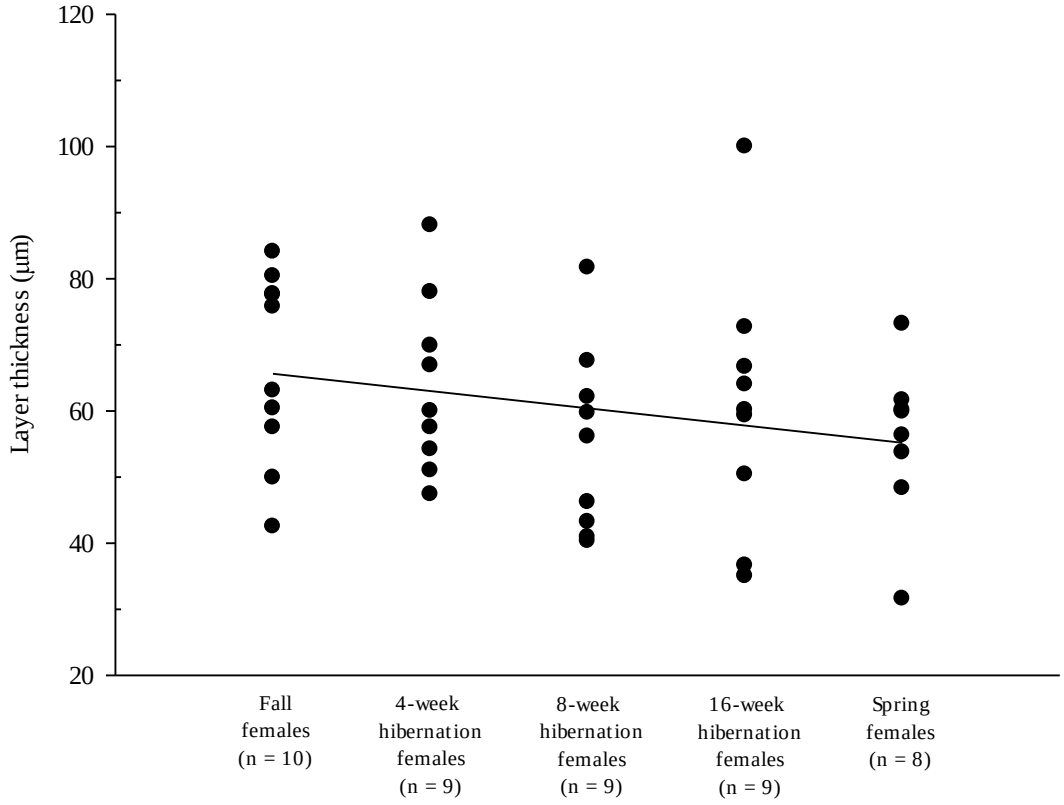

Figure 7: Regression of stratum germinativum layer thickness versus sampling period for female red-sided garter snakes. Samples were collected at five time points before, during, and following hibernation.

Discussion:

Of the forty five red-sided garter snake skin samples analyzed, I found a significant difference only in the thickness of the beta keratin layer across sampling periods. Females were found to have thinner beta keratin layers following spring emergence compared to at 4 weeks into hibernation and it was also demonstrated that the beta keratin layer progressively decreased in thickness across the hibernation period. In regards to the alpha keratin and stratum germinativum

layers, I did not observe a significant difference in thickness or a correlation across time period sampled.

Initially I hypothesized that an increase in the thickness of one of the layers would correlate with the time of pheromone expression because I assumed that the pheromone would be in the process of being produced at that time. If pheromones are produced by a particular layer, then an increase in thickness would represent more cells, or larger cells, being present in the layer to upregulate this activity. From the results, however, I did not see any significant increase in thickness across the three layers examined. This suggests that if there is an upregulation in pheromone production, which is thought to occur mid-way through hibernation (Parker and Mason, 2009), then the upregulation does not appear to correlate with an increase in cell size or number within the specific layers. Instead, cells that are already present may simply increase their rate of production of skin lipid components. This lack of cell growth and division during hibernation makes sense because while in hibernation the snakes must limit their energy expenditures due to lack of food.

I did observe a statistically significant decrease in the thickness of the beta keratin layer during hibernation which could be correlated to an increase in pheromone expression on the surface of the snake. In vertebrates, one of skin's main functions is to act as an impermeable barrier between the external environment and internal environment. For animals that use pheromones as a form of communication, there needs to be a way to transfer lipids from below the skin to the surface without destroying this barrier. Snakes might use a thinning of the outer layer of skin to allow more skin lipids to reach the surface quickly. As we see in my

results, the beta keratin layer decreases in thickness near the time period when pheromone expression is known to occur (Parker and Mason, 2009). A better understanding of the movement of skin lipids is necessary to fully understand this relationship.

Red-sided garter snake skin contains additional layers that were not measured in this project. One such layer is the oberhautchen layer which is found underneath the scale edge and is also composed of beta keratin. This layer has been linked to a system of micropores that could be involved in pheromone delivery (Lillywhite, 1982). If this layer had been measured, I might have seen a correlation between thickness and sampling period. Being composed of beta keratin this layer could also show the true importance of beta keratin cells during pheromone production by indicating if the decrease was across all beta keratin cell layers or just the layer forming the outside of the scales. Future studies are necessary to determine to what extent the oberhautchen layer changes thickness across the hibernation period.

The results of this research offers another window into understanding the mechanisms involved in pheromone communication in red-sided garter snakes. When exiting hibernation dens, these animals need to find a way to show their readiness for mating and have evolved a complex pheromonally-mediated communication system to accomplish this task. With skin being mostly impermeable to the passage of materials, changes must occur to allow chemicals produced deep in the skin to migrate to the surface. I suggest that changes in the relative thickness of

epidermal layers contribute to this phenomenon but more research needs to be done to support or refute this finding.

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