Spring 2017

Searching for sex differences in snake skin

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Searching for Sex Differences in Snake Skin

An Honors College Project Presented to

the Faculty of the Undergraduate

College of Science and Mathematics

James Madison University

by Sydney E. Ashton

May 2017

Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Honors College.

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PUBLIC PRESENTATION

This work is accepted for presentation, in part or in full, at annual meeting of the Society for Integrative and Comparative Biology in New Orleans, LA on Jan. 6, 2017.

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Acknowledgments

I would first like to thank my adviser and mentor, Rocky Parker for the time, resources and funding he devoted to this project and, more importantly, my development as a scientist and leader. I'd like to thank my thesis committee, Ray Enke and Bisi Velayudhan, for their support and advice throughout this research as well as the James Madison University Honors College for providing me with the framework for academic growth and achievement in my undergraduate career. Shannon Richard helped with animal collection, courtship trials, tissue collection and RNA extraction. Shannon Richard and Ricky Flores assisted with animal care. Ray Enke and Christy Schnitzler offered advice on initial stages of the design. Thanks to the James Madison University Department of Biology for providing resources for this research and Manitoba Conservation for allowing the field component of this work, including animal collection. I'd also like to thank Dave Roberts for providing logistical support in the field and Corey Cleland for influencing me to pursue research.

Abstract

The production of many secondary sexual signals, including pheromones, is controlled by sex hormone action at the sites of signal synthesis. The red-garter snake (*Thamnophis sirtalis parietalis*) is an ideal vertebrate for studying the interaction between steroids and sexual signals: males exclusively rely on skin-based female pheromones during courtship, and pheromone composition is augmented by treatment with sex steroids (e.g., males produce female pheromone if implanted with estrogen). But how do steroid hormones promote pheromone expression at the molecular level in snake skin? Feminizing effects of estrogens on sexual signals are known to result from activation of estrogen receptors α (Esr1) and/or β (Esr2), while masculinizing effects of androgens arise from androgen receptor (AR) activation. We hypothesized that Esr1 and Esr2 are expressed in garter snake skin but their expression is sex-dependent with female skin expressing higher levels of Esrs. To test this, red-sided garter snakes (n=7 males, n=7 females) were collected in the spring mating season, and mRNAs from skin and control tissues (liver) were extracted and used to synthesize cDNAs. Primers were designed using the available *T. sirtalis* genome (NCBI) and tested in real-time PCR reactions. While both receptor types were expressed in male and female skin, Esr1 was more highly expressed in female skin. We thus attribute the feminizing effect of estrogen on pheromone phenotype in males to their lack of circulating estrogen and subsequently dormant Esrs.

Introduction

i. Sexual signals in vertebrates

Sexual dimorphism, the expression of different characteristics between males and females of a single species, is ubiquitous throughout the major groups of vertebrates, from fish to birds. Male lions (*Panthera leo*), for example, have large manes that signal their dominance and age whereas females do not (West and Packer, 2002). Further, sexually dimorphic features may be used as sexual signals, allowing conspecifics to communicate with one another. These signals allow individuals to recognize conspecifics and the subsequent assessment of traits such as size, sex and body condition (McLean et al., 2012). Sexual signals can be morphological, behavioral or physiological. Morphological signals include characteristics such as body size or color, whereas behavioral signals include visual displays and acoustic signals (Andersson, 1994). Female poison frogs (*Dendrobatidae* family), for example, can be much larger than males (Shine, 1979), and male zebra finches (*Taeniopygia guttata*) have brightly colored beaks compared to females (McGraw, 2006). Both would be examples of morphological signals that can promote sexually dimorphic behavioral signals, such as calling behavior or courtship dances, respectively. Further, the expression of many sexual signals is controlled by sex steroid hormones. In the case of the zebra finch, for example, the beaks of testosterone-treated females will change from dull orange to the bright red, male-typical coloration and elicit interest from females (McGraw, 2006). Similarly, chemical signals allow conspecifics to communicate with one another, often via pheromones that exhibit pronounced sexual dimorphism.

Pheromones, which are used to facilitate reproduction in both vertebrates and invertebrates, can exist as single compounds or, more often, as blends of chemicals that differ between the sexes. An example of pheromone signaling in vertebrates comes from birds. Mallards (*Anas platyrhynchos*) use pheromones in mate choice and secrete these pheromones from the uropygial gland, a sebaceous gland present in most birds, that secretes waxes primarily used in waterproofing. Uropygial pheromone production in female mallards is mediated by estrogens. Moreover, males will produce the female typical blend of pheromone compounds when injected with estrogens (van Oordt, 1931; Caro et al., 2014). Goats (*Capra aegagrus*) experience a phenomenon known as the "male effect" in which a primer pheromone produced by males increases luteinizing hormone pulse frequency and induces estrus in females; females treated with testosterone will produce the primer pheromone and experience the characteristics associated with the male effect (Kakuma et al., 2007). Similarly, castrated geckos (*Goniurosaurus lichtenfelderi*) show decreased expression of male chemical cues, which returns to the male typical composition when castrated males are supplemented with testosterone (Golinski et al., 2015). Thus, sexual signals are not only sexually dimorphic in their expression, but their expression is typically mediated by sex steroid hormones.

Due to their lipid-soluble nature, sex steroid hormones, more specifically estrogens and androgens, cross the plasma membrane to enter the cell where they bind to specific receptors to cause hormone action. In the canonical pathway, estrogen receptors α and β (genes Esr1, and Esr2) and androgen receptor (AR) act as nuclear receptors that are activated by the binding of their cognate ligands, estrogens and

androgens, respectively. Activated nuclear receptors function primarily as transcription factors to control gene expression. Sex steroid hormones therefore affect target tissues in which their specific receptors are expressed. Generally, sex steroid hormones primarily target the brain and multiple reproductive tissues. In the green anole lizard (*Anolis carolinensis*), for example, AR is expressed in areas of the brain associated with reproductive behaviors (Rosen et al., 2002). One characteristic of the male copulatory system is the presence of two hemipenes (intromittent organs), expression of which is present in both embryonic males and females but completely regresses in females prior to hatching (Beck and Wade, 2005). Male embryos express higher levels of AR mRNA in the anterior segment of the tail than do females of the same age whereas females have higher expression of Esr1 mRNA in their tails than males (Beck and Wade, 2005). Therefore, expression of sex steroid receptors is sexually dimorphic in tissues associated with sexually dimorphic characteristics. In many vertebrates, the expression of sexually dimorphic pheromones occurs in the skin (Wyatt, 2003). Moreover, it has been shown that sex steroid receptors are expressed in several types of skin cells. In humans, AR is expressed in the keratinocytes of the epidermis and in both basal cells and sebocytes in sebaceous glands. Esr1 is expressed in sebocytes, and Esr2, like AR, is expressed in the epidermis as well as the basal cells and sebocytes of sebaceous glands (Pelletier and Ren, 2004). However, sexual dimorphisms in the expression of sex steroid receptors in skin have not been studied, especially in vertebrates that use sexual signals arising from the skin.

ii. *Thamnophis sirtalis parietalis* and its sex pheromones

Our model vertebrate, *Thamnophis sirtalis parietalis,* the red-sided garter snake, is a well-studied subspecies of the common garter snake, the most widespread reptile in the western hemisphere. During the breeding season (April-May) each year, thousands of red-sided garter snakes emerge from their winter hibernacula in a phenomenal natural event that has been the focus of intense, diverse research for more than 40 years (Mason, 1993). During the spring breeding season, males in this system emerge first and remain in the general vicinity of the den site while females emerge singly throughout the season, resulting in a significantly male-biased sex ratio at the den (Gregory, 1974). As each female emerges, she is instantly courted by a large number of males in what is termed a mating ball (Crews and Gartska, 1982). The males court unmated, sexually attractive females using behaviors only observed in a mating context, beginning with the males pressing their chins onto the female's back accompanied by rapid tongue-flicking, followed by the male moving up and down the female's body and eventually coming to rest with his head behind that of the female's at which point he exhibits caudocephalic waves (Mason, 1993). The male red-sided garter snake uses a sexually dimorphic pheromone to distinguish females from males (Mason et al., 1989; Mason et al., 1990; Mason and Parker, 2010). The female sexual attractiveness pheromone is a blend of compounds that is distinct from that produced by males and also conveys information pertinent to male mate-choice, such as size and body condition (LeMaster and Mason, 2002; Shine et al., 2003).

Recent work discovered that the sex pheromone is controlled by sex steroid hormones. Males, when implanted with estrogen, can be stimulated to produce the

sexual attractiveness pheromone and become extremely attractive to, and will subsequently be courted by, wild males (Parker and Mason, 2012). Furthermore, castrated males also produce the female pheromone blend and are courted by wild males (Parker and Mason, 2014). However, similar to the gecko, the pheromone composition returns to the male typical blend when castrated males are supplemented with testosterone, and these snakes are once again not attractive to wild males (Parker and Mason, 2014). Thus, sexually dimorphic pheromone expression in the skin of the garter snake is regulated by sex steroid hormones. An outstanding question remains: how can steroids augment pheromone expression at the proximate level? Where are their hormone receptors? The purpose of my thesis was to determine if there are innate sexual dimorphisms in the expression of estrogen receptors in garter snake skin. I hypothesized that female garter snake skin would show increased levels of Esr expression relative to males during the spring mating season. I tested this hypothesis by utilizing quantitative PCR (qPCR) to assess expression levels of estrogen receptors α and β in isolated tissues collected from red-sided garter snakes.

Materials and methods

i. Animal collection

Male and female red-sided garter snakes were collected from the den site in Inwood, Manitoba, Canada, in May 2016 during the spring mating season. Females were collected immediately upon emergence from the hibernaculum and checked for the absence of a copulatory plug to ensure that they were unmated. Actively courting males were collected from natural mating balls in and around the den. Courtship bioassays were performed to verify that females were attractive and that males were both unattractive and actively courting. One female was placed in a nylon arena with ten actively courting males to simulate a natural mating ball. Females were only selected for RNA extraction if at least 80% of males reached a 2 or higher on the ethogram in each of three mating balls (Table 1). Males were only selected if they displayed chin rubbing behavior on an attractive female and were not the object of any courtship behavior from other males. The snout-vent-length (SVL) and body mass of each animal were recorded, and the ratio of SVL:mass was used to exclude animals with very high and very low body conditions (Figure 1). Selected animals (n=7/sex) were returned to the laboratory and housed in 10 gallon glass aquaria within an environmental chamber set to mimic Manitoba spring conditions (16 h:8 h L:D; 25:15°C). Water was provided *ad libitum* but food was not provided as snakes do not eat during the breeding season.

All field and laboratory procedures involving the use of vertebrate animals were approved by the IACUC at James Madison University (protocol #A16-03 and A16-14).

Table 1. Ethogram of red-sided garter snake courtship behavior (Crews et al. 1984; Moore et al., 2000; LeMaster, 2002). Scores of 2 and higher are only observed in a mating context.

Score	Description
1.0	Male investigates female, increased tongue-flick rate
2.0	Male chin rubs female with rapid tongue- flicks
3.0	Male aligns body with female
4.0	Male actively tail searches and attempts cloacal apposition and copulation with female; possible caudocephalic waves
5.0	Male copulates with female

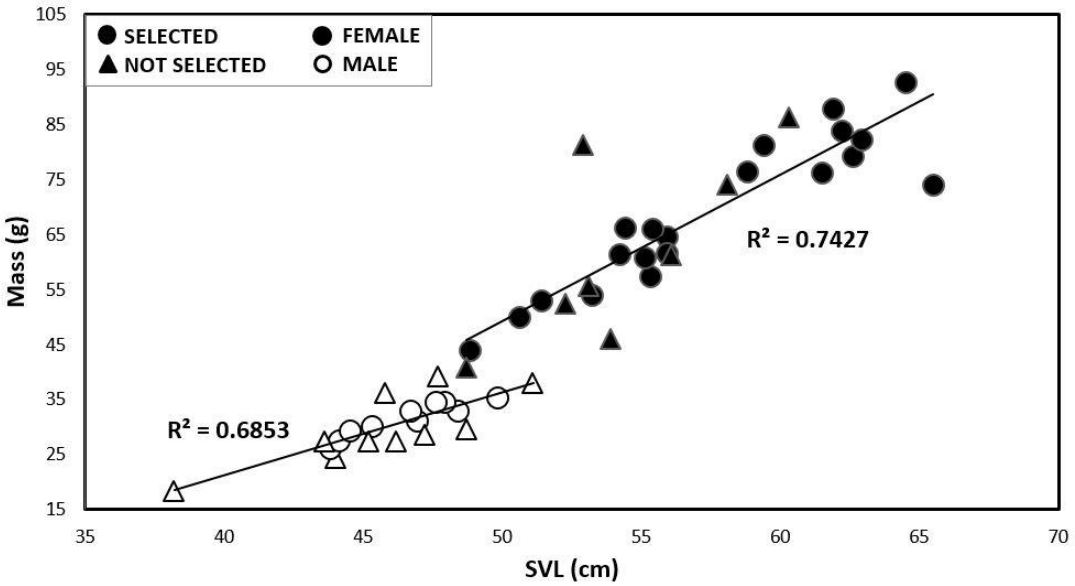

Fig 1. Garter snakes were selected for RNA extraction based on condition and attractiveness (females) or courtship behavior (males). Selected females were courted by >80% of males in the field, and all males were actively courting females in the spring 2016. Note the female-biased sexual size dimorphism.

ii. RNA extraction and cDNA synthesis

Animals were euthanized with an overdose of sodium brevital (0.3 ml of 1% brevital in reptile Ringers). Sections (approximately 0.5x1 mm) from the following tissues were then collected and immediately snap frozen in liquid nitrogen: dorsal skin, liver and testis/ovary. Tissues were stored at -80°C until use. To extract RNA, each tissue sample was individually pulverized in a Cryo-Cup grinder; methods then followed PureLink RNA Mini Kit (Life Sciences) instructions, including on-column DNaseI treatment (Life Sciences). Quality and concentration of RNA was checked (NanoDrop; 260nm/280nm ratio) and a standardized amount of RNA (0.2 µg per tissue) was used in cDNA synthesis (random primers; SuperScript VILO kit) in order to control for cDNA concentration. cDNA quality was finally checked by running 2% agarose gels of RT-PCR products with control primers (see below).

iii. Primer design

Primers were designed for Esr1, Esr2 and Gapdh, a control gene constitutively expressed in all cells, from *T. sirtalis* sequences obtained from NCBI (http://www.ncbi.nlm.nih.gov/). First, protein alignments for each gene of interest were performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and NCBI protein sequences for various vertebrates both closely and very distantly related to the garter snake (Figure 2). Highly conserved regions of each protein were targeted for primer design using NCBI's Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Furthermore, primer sets were designed to span putative exon-exon junctions based on known intron locations within the chicken (*Gallus gallus*) genome and, when primers were run in NCBI's Primer-BLAST, only *T. sirtalis* was returned for each gene. Overall, 50 primer pairs were designed and tested (Table 2). Of the sets of primers that consistently resulted in single bands at the predicted size, one pair was selected for each gene of interest for use in PCR experiments (Table 3).

a.

```
ZebraFish       AFFKRSIQG--HNDYVCPATNQCTIDRNRRKSCQACRLRKCYEVGMMKGGIRKDRGGRSV
Frog            AFFKRSIQG--HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRM
Mouse           AFFKRSIQG--HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRM
Rabbit          AFFKRSIQGNRHNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRM
Human           AFFKRSIQG--HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRM
Pig             AFFKRSIQG--HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRM
Goat            AFFKRSIQG--HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRM
Cow             AFFKRSIQG--HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRM
Turtle          AFFKRSIQG--HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRM
Alligator       AFFKRSIQG--HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRM
Ostrich         AFFKRSIQG--HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRM
Chicken         AFFKRSIQG--HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGEM
Mallard         AFFKRSIQG--HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRM
Anole           AFFKRSIQG--HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRM
GarterSnake     AFFKRSIQG--HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRI
RatSnake        AFFKRSIQG--HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRI
                ********  ****:**********:**************************** *  :
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b.

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ZebraFish       DGAYEYPNPTQ-TFGTSS--------PA-EPASVGYYPAPP-----DPHEEHLQTLGGGS
Frog            TGIFNYPEGTTYDFAA-------AAAPVYSSASLSYAASSE--TFGSSSLTGLHTLNNVP
Mouse           PTVFNYPEGAAYEFNAAAAAAAAAASAPVYGQSGIAYGPGSEAAAFSANSLGAFPQLNSVS
Rabbit          PAVYNYPEGAAYEFNAAAAAAAAAATAPVYSQSGLAYGPGSEA-AFGSNGLGGFPQLNSVS
Human           PAVYNYPEGAAYEFNAAAA----ANAQVYGQTGLPYGPGSEAAAFGSNGLGGFPPLNSVS
Pig             PAVYNYPEGAAYDFNAAAA----ASAPVYGQSGLAYGPGSEAAAFGANGLGGFQPLNSVS
Goat            PAVYNYPEGAAYDFNAAAA----ASAPVYGQSGLPYGPGSEAAAFGANGLGAFPPLNSVS
Cow             PAVYNYPEGAAYDFNAAAP----ASAPVYGQSGLPYGPGSEAAAFGANGLGAFPPLNSVS
Turtle          TGVFNYPEGTTYDFAT--------AAPVYSSTSLSYAPASE--SFGSSSLGGFHSLNNVP
Alligator       TGIFNYPEGTTYDFAT--------AAPVYSSTSLSYAPTSE--SYGSSSLGGFHSLNNVP
Ostrich         TGVFNYPEGTAYDFAT--------TAPVYSSTTLSYAPTSE--SFGSSSLAGFHSLNNVP
Chicken         TGVFNYPEGATYDFGT--------TAPVYGSTTLSYAPTSE--SFGSSSLAGFHSLNNVP
Mallard         TGVFNYPEGATYDFGT--------TAPVYGSTTLSYAPTSE--SFGSSSLAGFHSLNNVP
Anole           TGVFNYPEGSVYDFST--------AAPVYSSTSLTYAPTTE--SFGSNSLGGFHSLNNVP
GarterSnake     ------------------------------------------------------------
RatSnake        TGGFNYPDGAAYEFST--------AAPVYSSTSLSYPPTSE--SFGTSSLGGFHSLNNVP
```

Figure 2. Sample Esr-α protein alignments between the garter snake, *T. sirtalis,* and 15 other species. Symbols as the bottom of each set represent the consensus, with an asterisk indicating full conservation for that residue. Some sections of the garter snake Esr-α protein are highly conserved (a), while some chunks of the sequence are missing from the garter snake genome entirely (b).

Table 2. A total of 50 primer pairs were designed and tested with a 26% success rate (green cells).

	Amplicon size			
	<120 bp		>300 bp	
Esr1	4	11	1	1
Esr2	3	12	1	1
GAPDH	3	11	1	1

Table 3. Amplicon length, sequences and melting temperatures for primers used in semi-quantitative and quantitative RT-PCR experiments.

Gene	Size (bp)	Forward primer (5'-3')	Tm	Reverse primer (5'-3')	Tm
Esr1	112	TGACCCTAACAGACCCTTCAAC	59.63	CACAAAGCCTGGAACCCTTTTG	60.48
Esr2	118	CACATCTCTCCTCTGACAGTGC	60.42	AGGTGTCTCTGTGAATAGGCAAG	60.06
Gapdh	101	TGACTCTACTCATGGCCGTTTC	60.09	CAGGATCACGCTCTTGGAAAAC	59.84

iv. PCR

cDNA quality was first assessed by semi-quantitative RT-PCR (Platinum Taq; Life Sciences), with Gapdh serving as the control gene (Table 4). Reactions with no reverse transcriptase comprised the negative control and amplification products were visualized with gel electrophoresis (2% agarose gels, 130 V for 1 hour, 5 µL per sample). Liver served as a positive control. I used densitometric analysis with ImageJ to measure the integrated pixel density of each band on the gel, relativizing skin expression to liver for each individual; a single individual's skin and liver amplifications were run on the same gel, and each gel contained both a male and a female in order to minimize the effects of gel variation. This served as an approximation of relative expression levels before moving to a quantitative approach. Quantitative RT-PCR (qPCR) (Sybr Green; ThermoFisher) was then performed using the $2^{-\Delta\Delta Ct}$ method with Gapdh as a control gene and liver the control tissue (Pffafl, 2001; see Figure 3 for plate design and Table 5 for conditions).

Table 4. Semi-quantitative (left) and quantitative (right) RT-PCR cycle. Products (5 µL per sample) were run on a 2% agarose gel with 1.5 µL of a 1kb DNA ladder.

	Temp (°C)	Time (m:s)
	94	01:00
40x	94	00:30
	60	00:30
	68	00:30
	68	07:00
	4	Pause

	Temp (°C)	Time (m:s)
	95	0:30
45x	95	0:05
	60	00:30
	65	0:31
Melt curve	65 to 95	0.5 C increment for 0:05

		F1 Esr1			F1 Gapdh			M1 Esr1			M1 Gapdh		
Skin	1:1	○	○	○	○	○	○	○	○	○	○	○	○
	1:10	○	○	○	○	○	○	○	○	○	○	○	○
	1:100	○	○	○	○	○	○	○	○	○	○	○	○
	1:1000	○	○	○	○	○	○	○	○	○	○	○	○
Liver	1:1	○	○	○	○	○	○	○	○	○	○	○	○
	1:10	○	○	○	○	○	○	○	○	○	○	○	○
	1:100	○	○	○	○	○	○	○	○	○	○	○	○
	1:1000	○	○	○	○	○	○	○	○	○	○	○	○

Figure 3. Plate design for qPCR experiments. Each 96 well plate contained four dilutions of both skin and liver cDNA run with a gene of interest and the control gene, Gapdh, for one female and one male.

Results

i. Semi-quantitative RT-PCR

Esr1, Esr2 and Gapdh were all expressed in the skin and liver of both males and females (Figures 4 and 5). All amplicons were of predicted band size. Using ImageJ to approximate optical density of each band relative to Gapdh for that individual revealed that, though expressed in both sexes, Esr1 may be more highly expressed in female skin, though the difference is not significant ($p=0.12$; Figure 5). Variation between male and female Esr2 expression was also not significant ($p=0.28$; Figure 5). Interestingly, there was a high amount of variation of Esr1 expression in female skin as compared to males and Esr2 expression in both sexes.

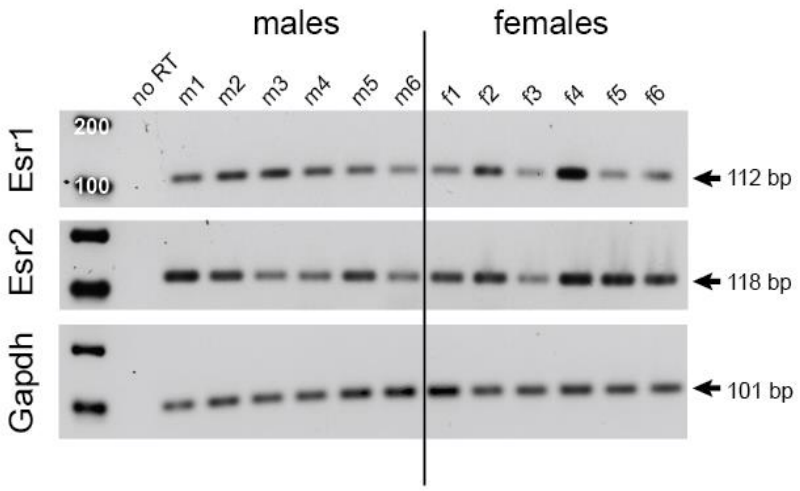

Fig. 4. Semi-quantitative RT-PCR amplification products run on 2% agarose gel (inverted) using dorsal skin cDNA. Each band represents a single individual.

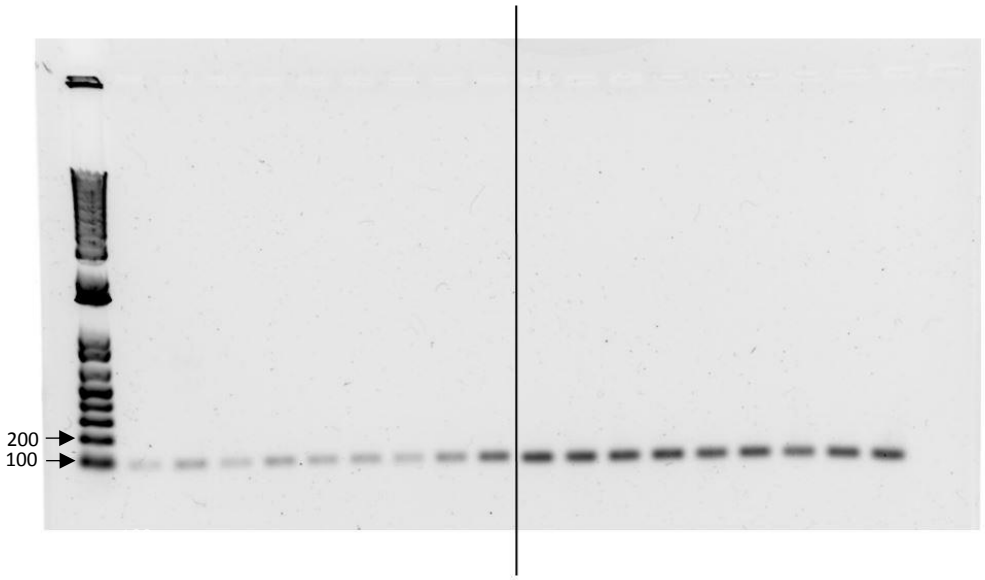

Fig. 5. Liver expression of Esr1 (lanes 2-10) and Gapdh (lanes 11-19) for n=6 females and n=3 males. RT-PCR products were electrophoresed on 2% agarose gel.

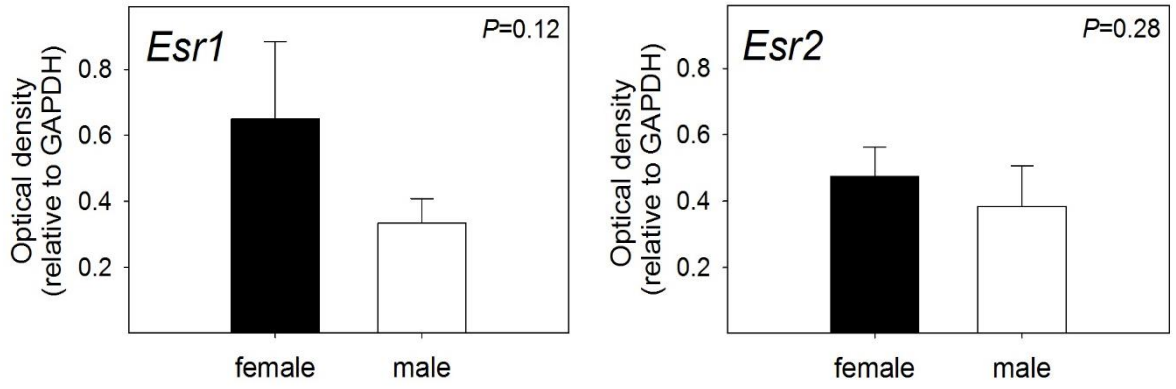

Fig. 6. Relative band densities (vs. Gapdh) for each individual as determined via ImageJ. Bars represent means + SEM (n = 7/sex)

ii. Quantitative RT-PCR

There were low to undetectable rates of primer dimerization and melt curves showed only one peak (Figure 6). For Esr1, the data were not normally distributed so I used a Mann-Whitney rank sum test (nonparametric t-test). Expression was significantly different between male and female skin relative to liver for Esr1 ($U=5.00$, $P=0.041$). Esr2 data were normally distributed, so a t-test was used to analyze the difference in relative expression between male and female skin for Esr2. Expression was not significantly different for Esr2 ($t=0.196$, $P=0.848$; Figure 7).

Because estrogen receptors were underexpressed in female liver, statistical analyses were repeated correcting for this underexpression. Esr1 data were not normally distributed so a Mann-Whitney rank sum test was used (nonparametric t-test). Expression was significantly different between male and female skin relative to liver for Esr1 ($U=4.00$, $P=0.026$). For Esr2, the data were normally distributed, so a t-test was used to analyze the difference in relative expression between male and female skin for Esr2. Expression was not significantly different for Esr2 ($t=1.15$, $P=0.276$).

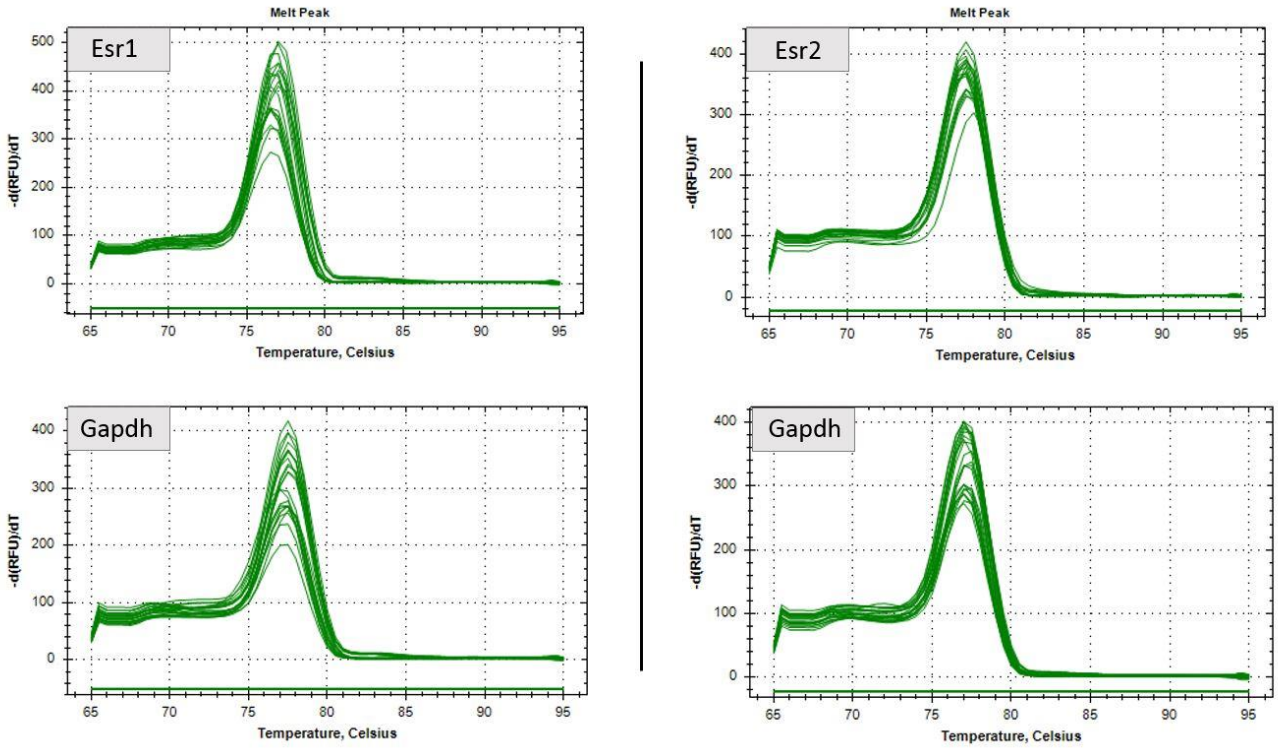

Fig. 7. Melt curves from Esr1 (left) and Esr2 (right) qPCR experiments. Each graph represents a single individual and includes curves from a range of dilutions and both skin and liver cDNA as according to the plate design outlined in Fig. 3.

Fig. 8. Expression of Esr1 and Esr2 in male and female garter snake skin relative to both GAPDH and liver. Bars represent means + SEM (n=6/sex). A fold-change value of one indicates that expression is equal in skin and liver.

Discussion

During the spring mating season, female red-sided garter snakes secrete from their skin a pheromone blend that elicits courtship behavior in males (Mason et al., 1989; Mason et al., 1990; Mason and Parker, 2010). The production of this pheromone is mediated by estrogen and, consequently, males treated with estrogen will be courted by wild males following hibernation (Parker and Mason, 2012). It is hypothesized that pheromone synthesis occurs within the skin because garter snakes do not have skin glands, and it follows that pheromone is produced locally at the site where it is sensed by males (Gartska et al. 1982; Mason et al. 1989). Importantly, both quantitative (qPCR) and semi-quantitative (RT-PCR/ImageJ) analysis show that Esr1 and Esr2 are expressed in both female and male garter snake skin. I suggest that these skin receptors activate the canonical estrogen signaling pathway to control pheromone production.

Relative to liver expression levels, Esr1 is enriched in female skin. In males, Esr1 is more highly expressed in liver than in skin. It is important to note that there is a high level of variation in Esr1 expression, particularly among females. The pheromone blend produced by female red-sided garter snakes varies widely across individuals and is affected by traits including size and body condition (LeMaster and Mason 2002). Female pheromone composition has additionally been shown to vary with respect to time following emergence from hibernacula, mirroring the decline in estradiol post-emergence (Uhrig et al. 2014). Moreover, treatment of females with estradiol benzoate only increases their attractivity if performed within specific points of the shedding cycle (Kubie et al. 1978). Thus, pheromone composition is dynamically influenced by

estrogen. The variation in Esr1 expression may alter a female's sensitivity to estrogen, augmenting the composition of her pheromone blend and thus her level of attractivity. The sexual dimorphism apparent in Esr1 expression disappears for Esr2, which also shows less variation among both sexes. Esr2 is expressed similarly in female skin and liver and is even more underrepresented in male skin versus liver than is Esr1. These data suggest that Esr1 may play a more prominent role in skin estrogen-signaling than Esr2 during the spring mating season and may be responsible for the individual variation in pheromone composition.

Viviparous squamates, including garter snakes, undergo vitellogenesis during which the liver synthesizes proteins necessary for yolk development, primarily vitellogenin (Garstka, 1985). This protein synthesis causes enlargement of the liver and is controlled by estrogens. For example, when male zebra fish (*Danio rerio*) are treated with EE2, which is the synthetic form of estradiol used in contraceptives, blood vessels in the liver dilate, and differential staining of liver sections indicates high vitellogenin content relative to control males (Van den Belt et al*.* 2002). Similarly, previous work on red-sided garter snakes found that liver mass and blood viscosity increased in males after treatment with estradiol (Garstka and Crews 1981; Parker and Mason 2012). This evidence supports estrogen receptor activity in the liver of both male and female garter snakes, allowing for its use as a control tissue against which skin expression was compared using the $2^{-\Delta\Delta Ct}$ method. This method, however, does not account for potential sexual dimorphisms of Esr expression in the liver.

Surprisingly, estrogen receptors were underexpressed in the female liver during the spring mating season. We repeated statistical analysis of qPCR data correcting for

this and found that the sexual dimorphism of Esr1 was still significant and the Esr2 relationship remained non-significant. Taken together, this evidence suggests that Esr1 may have a role in the hormonally-mediated expression of the sexual attractiveness pheromone in the red-sided garter snake. Further research is necessary to elucidate the nature and extent of this role and identify how Esr2 may be involved. Future experiments should aim to assess the seasonal effect on sex steroid receptor expression, including AR which recognizes androgens such as testosterone. Though courtship occurs in the spring, which was the season I focused on for this study, garter snakes demonstrate a dissociated breeding season; that is, their reproductive behaviors do not coincide with high levels of circulating sex steroid hormones (Crews et al., 1984). Moreover, treating male garter snakes with sex steroid or other hormones is insufficient to stimulate courtship behaviors (Camazine et al., 1980; Crews et al., 1984). Because circulating levels of androgens and estrogens are elevated in the fall prior to hibernation (Krohmer et al., 1987; Lutterschmidt and Mason, 2009), sex steroid hormone receptor expression may also be seasonally variable. When tissues were collected for RNA extraction in the present study, tissues (skin, liver, gonads, duodenum) were also fixed and sectioned for use in immunohistochemistry (IHC) according to the methods in Parker et al. 2014. IHC will allow for the visualization of receptor protein expression in the skin, which would complement PCR results, and thus the investigation of receptor localization with respect to sex and season. These additional studies are necessary to better understand pheromone production in the garter snake and would propel our knowledge of the hormonal control of sexual signals.

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