J Chem Ecol (2012) 38:71–80
DOI 10.1007/s10886-011-0054-x

Pheromonal Mediation of Intraseasonal Declines in the Attractivity of Female Red-Sided Garter Snakes, *Thamnophis sirtalis parietalis*

Emily J. Uhrig · Deborah I. Lutterschmidt · Robert T. Mason · Michael P. LeMaster

Received: 22 July 2011 / Revised: 6 November 2011 / Accepted: 19 December 2011 / Published online: 10 January 2012

Abstract During the breeding season, female red-sided garter snakes (*Thamnophis sirtalis parietalis*) produce and express a sexual attractiveness pheromone that elicits male courtship behavior. Composed of a homologous series of saturated and monounsaturated methyl ketones, this pheromone is expressed in female skin lipids. Recent studies have shown that the sexual attractivity of unmated female garter snakes declines as the breeding season progresses. Here, we investigated whether temporal changes in the quantity and/or quality of the female sexual attractiveness pheromone are responsible for the observed loss of attractivity. Female red-sided garter snakes were collected immediately following spring emergence and held under natural conditions for the duration of the breeding season. Behavioral experiments confirmed that unmated females become significantly less attractive to males within two weeks of emergence from hibernation. Additionally, these females had lower estradiol concentrations at two weeks post-emergence. Subsequent chemical analyses revealed qualitative variation between the pheromone profiles of newly emerged females and those of females at two weeks post-emergence. Together, these results support the hypothesis that changes in the female sexual attractiveness pheromone are responsible for declining post-emergence female attractivity in garter snakes.

E. J. Uhrig · M. P. LeMaster
Department of Biology, Western Oregon University,
Monmouth, OR 97361, USA

D. I. Lutterschmidt
Department of Biology, Portland State University,
Portland, OR 97207, USA

E. J. Uhrig (✉) · R. T. Mason
Department of Zoology, Oregon State University,
Corvallis, OR 97331, USA
e-mail: uhrige@science.oregonstate.edu

Key Words Pheromone · *Thamnophis sirtalis parietalis* · Red-sided garter snake · Reptile · Courtship behavior · Methyl ketones · Estradiol

Introduction

Mate attraction is often a costly endeavor that may be energetically expensive (Vehrencamp et al., 1989) or increase an individual's risk of mortality (Mougeot and Bretagnolle, 2000; Shine et al., 2004). Thus, for many species, attracting potential mates on a constant basis would not be beneficial, particularly at times when environmental conditions are less favorable for reproduction (e.g., colder temperatures, less abundant food resources). Mate attraction, therefore, tends to be limited to discrete breeding seasons with reproductive signals varying seasonally such that individuals attract the opposite sex only at certain times of the year. For example, many vertebrates vary their physical appearance [e.g., American goldfinch (*Carduelis tristis*; McGraw, 2004); striped plateau lizard (*Sceloporus virgatus*; Weiss, 2006)] or behavior patterns between breeding and non-breeding seasons [e.g., green anole lizard (*Anolis carolinensis*; Neal and Wade, 2007); golden-collared manakin (*Manacus vitellinus*; Day et al., 2007)].

Interseasonal variation also occurs with respect to chemically-mediated mate attraction as a number of species vary the concentration and/or composition of chemical signals between breeding and non-breeding seasons [e.g., Australian tree frog (*Litoria splendida*; Wabnitz et al., 2000); red-sided garter snake (*Thamnophis sirtalis parietalis*; LeMaster and Mason, 2001a); ringtailed lemur (*Lemur catta*; Scordato et al., 2007)]. Changes in vertebrate chemical signals that occur intraseasonally, such as during the course of a single breeding season, are more poorly

understood. Nevertheless, intraseasonal variation in chemical signaling can have important implications for signal producers by helping to further minimize the time they are subject to fitness costs associated with mate attraction.

The red-sided garter snake presents an opportunity to study intraseasonal variation in a vertebrate pheromone. The female sexual attractiveness pheromone of this species has been identified chemically as a homologous series of long-chain saturated and monounsaturated methyl ketones (Mason, 1992, 1993), thus allowing for both quantitative and qualitative chemical comparisons. Further, mate recognition relies extensively upon male detection of the pheromone, which elicits stereotypical male courtship behaviors that only occur in a reproductive context and are easily recognized by observers (Crews et al., 1984; Mason et al., 1989; LeMaster and Mason, 2002). Production of the pheromone, which is sequestered within the skin lipids of the female's dorsal surface, appears to be under estrogen control, as exogenous estrogen treatment induces both attractivity in females and expression of female-like pheromone profiles in males (Crews, 1976, 1985; Kubie et al., 1978; Parker, 2010). Previous studies have found the chemical profile of this pheromone to exhibit interseasonal variation, as well as both inter- and intrapopulational variation (LeMaster and Mason, 2001a, 2002, 2003). Intraseasonal variation has been hypothesized to occur (O'Donnell et al., 2004; Shine et al., 2005a), but to date no studies have investigated this hypothesis.

In a series of behavioral experiments, Shine et al. (2003a, 2005a) and O'Donnell et al. (2004) observed that female red-sided garter snakes, including unmated females, are less attractive to males as time progresses following emergence from hibernation. Shine et al. (2005a) demonstrated that the apparent decline in attractivity occurs relatively rapidly and independently of changes in male behavior. Further, Shine et al. (2005a) tested male courtship responses to isolated female skin lipid extracts and found males to exhibit more intense courtship toward the lipids of females newly emerged from hibernation. Based on their respective behavioral observations, both Shine et al. (2005a) and O'Donnell et al. (2004) suggested that changes in the chemical profile of the female sexual attractiveness pheromone during the course of the breeding season could be responsible for declining female attractivity. However, no chemical analyses were performed in either of these studies, and it has yet to be confirmed that the pheromone indeed exhibits intraseasonal variation.

Our study investigated whether the chemical profile of the red-sided garter snake sexual attractiveness pheromone exhibits the intraseasonal variation necessary to account for the observed decline in female attractivity. To test this hypothesis, we first performed behavioral experiments to confirm there was a decrease in attractivity. Subsequently, we conducted chemical analyses of the sexual attractiveness pheromone to examine potential changes in its quantity and/or quality. Given the apparent role of estrogen in pheromone expression, we hypothesized that pheromonal changes would be accompanied by hormonal variation; thus, we also assayed plasma estradiol concentrations. If the loss of attractivity results from changes in the sexual attractiveness pheromone profile, then we would expect a correlation between male behavior and variation in (1) overall pheromone concentration and/or (2) concentration of individual pheromone components when compared between newly emerged and post-emergence females. Further, as pheromone expression is believed to be estrogen regulated, we would expect the decline in female attractivity to be associated with decreased estradiol concentrations.

Methods and Materials

Study Population Red-sided garter snakes (*Thamnophis sirtalis parietalis*) were collected during the 2003 spring breeding season in the Interlake region of Manitoba, Canada (50°31'58"N; 97°29'71"W). The field site, located in an abandoned gravel quarry, contains an underground hibernaculum where approximately 35,000 garter snakes spend the winter months (Shine et al., 2006). Spring emergence marks the beginning of an annual cycle similar to that of other local populations of the species: participation in a brief mating period at the den site, migration to the summer feeding grounds, and then an autumn return to the den (Gregory, 1977).

Experimental Animals All female garter snakes utilized were collected immediately upon emergence from the hibernaculum to ensure they were unmated. Also, as pheromone composition is correlated with female body size (LeMaster and Mason, 2002), females of similar snout-vent length (SVL) were utilized (SVL: 60–66 cm). At the beginning of our experiment (week zero), a group of females (N=27) was collected and subsequently divided into 3 treatment groups corresponding to 3 time points: week zero, week one, week two. One group, week zero newly emerged females (N=8), were immediately bled for hormone sampling and then euthanized for pheromone collection. Females in the other treatment groups, hereafter referred to as 1 wk post-emergence females (N=9) and 2 wk post-emergence females (N=10), were kept in outdoor arenas [1×1×1 m; constructed of nylon cloth (Moore et al., 2000)] for 1 and 2 wk, respectively, at which time they were tested in behavioral trials. Additional newly emerged females, referred to as week one and week two newly emerged females, were collected at the week one and week two time points (N=7 and 9, respectively) and tested in behavioral trials on

those days. These newly emerged females served as controls to demonstrate that female attractivity declines independently of changes in male behavior.

Immediately prior to behavioral experiments, groups of actively courting males (~500 / group) were indiscriminately collected from the hibernaculum. Subsets of these males were randomly selected and utilized in behavioral trials the day of their collection, marked with a non-toxic pen, and released back at the den upon completion of that day's experiments. Thus, a new group of males was used at each time point. All procedures used in this study were approved by the Oregon State University Animal Care and Use Committee (protocol number: 2661) and the Manitoba Wildlife Animal Care Committee (protocol number: 2002–06). This research complied with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was carried out under the authority of Manitoba Wildlife Scientific Permit WSP 03009.

Courtship Trials Courtship trials were carried out in the previously described arenas which have been used extensively for similar behavioral studies within this species (e.g., Mason and Crews, 1985; Moore et al., 2000; Shine et al., 2000a; LeMaster and Mason, 2002). To limit confounding effects due to weather conditions, an array of ten arenas was set up, allowing multiple trials to be conducted simultaneously. Behavioral trials were performed on 2 d separated by a 1 wk interval: week one (N=16 trials: 7 trials of week one newly emerged control females; 9 trials of 1 wk post-emergence females), and week two (N=19 trials: 9 trials of week two newly emerged control females; 10 trials of 2 wk post-emergence females). For each behavioral trial, 10 actively courting males were placed into an arena and allowed to acclimate for 5 min. A female, randomly-selected from one of the appropriate treatment groups, then was placed into the arena. Snakes were allowed to interact undisturbed for 5 min after which time the number of males actively courting the experimental female was counted by an observer blind to the treatment. To be considered actively courting, a male had to be observed chin-rubbing or tail-searching along the dorsum of the female. These behaviors are seen only in a reproductive context in this species and are thus indicative of male sexual behavior (Crews et al., 1984).

The behavioral methods utilized in this study were similar to those used by Shine et al. (2005a) with modifications to allow for pheromone collection. First, we attempted to prevent mating, as it is known to immediately reduce female attractivity via the male's deposition of a copulatory pheromone (Whittier et al., 1985; Shine et al., 2000b; Mendonça and Crews, 2001). The presence of the copulatory pheromone potentially could confound behavioral and chemical analyses, thus mating was prevented by placing adhesive tape over the cloaca of each female and limiting the interaction time to a single 5 min period. Despite these precautions, two matings occurred during the experiments. The mated female trials, both in the 1 wk post-emergence treatment group, were excluded from all subsequent statistical analyses. Second, whereas Shine et al. (2005a) conducted simultaneous choice tests, males in each trial of our study were presented with a single female to prevent the potential intermingling of pheromones from the different female treatment groups.

Hormone Analysis: Blood Sampling and Radioimmunoassay Blood samples (approximately 200 μl; collected with heparinized 1-cm^3 syringes and 25-g needles) were obtained from the caudal veins of zero week newly emerged females (N=8), 1 wk post-emergence females (N=7), and 2 wk post-emergence females (N=10); 1 wk and 2 wk post-emergence samples were collected immediately following behavioral trials. The two mated females were excluded from the hormone analysis. Blood samples were stored on ice until return to the field station, where they were centrifuged to separate the plasma. Plasma samples then were stored at −4°C until return to Oregon State University, where they were stored at −80°C until further analysis.

The plasma samples were analyzed for estradiol concentrations following direct radioimmunoassay procedures described in detail by Lutterschmidt et al. (2004) and Lutterschmidt and Mason (2005). The methods used for direct radioimmunoassay of plasma estradiol were previously validated for female red-sided garter snakes by Lutterschmidt and Mason (2009). Briefly, 100 μl of each plasma sample were spiked with 2,000 cpm of tritiated steroid (Amersham Biosciences, Piscataway, NJ, USA) and incubated for 18–24 hr to determine individual sample recovery. Steroids were extracted twice from each plasma sample with anhydrous ethyl ether. The ether phase was removed and dried under nitrogen gas in a warm (37°C) water bath. Hormone extracts then were reconstituted in 500 μl of phosphate-buffered saline and incubated overnight at 4°C. To determine the percent hormone recovery for each plasma sample, a 50-μl aliquot was removed from each reconstituted sample and placed in a scintillation vial. Following incubation in toluene-based scintillation fluid for 12 h, the radioactivity of each sample was quantified in a Beckman LS 1800 scintillation counter. The resulting data were compared to the radioactivity of the initial spike of tritiated steroid and used to calculate the percent sample recovery obtained during the extraction procedure for each individual plasma sample.

The remainder of each reconstituted sample was allocated to two duplicate culture tubes for assay. Serial dilutions of the standard curve (performed in triplicate), 0% bound (or non-specific binding), 100% bound, and all samples were incubated with 12,000 cpm tritiated steroid (2,4,6,7,16,17-[^{3}H]Oestradiol, Amersham Biosciences, Piscataway, NJ, USA). Samples and maximum binding tubes also were incubated with 100 μl

of antiserum at 4°C for 18–24 h (estradiol antibody E-6006 from Wein Laboratories, Inc., Succasunna, NJ, USA). Unbound steroid was separated from bound hormone using dextran-coated charcoal. The bound steroid was decanted into scintillation vials, incubated in toluene-based scintillation fluid, and the radioactivity of each sample quantified in a scintillation counter.

All estradiol samples were analyzed in a single hormone assay. Mean sample recovery was 72% (± 1.9 SE), well within the range of accepted estradiol extraction efficiencies (e.g., Lynch et al., 2006; Taylor et al., 2004; Almli and Wilczynski, 2009; Lutterschmidt and Mason, 2009; Lutterschmidt et al., 2009). All hormone concentrations were corrected for individual recovery variation. The intra-assay coefficient of variation was 7.7%. Limits of detectability were approximately 1 pg/ml for estradiol. In some instances (<14% of the samples), estradiol concentrations were below the limits of detectability. To retain these samples in our statistical analyses, and because of the high sensitivity of our estradiol assay, we assigned each undetectable plasma sample the limit of detectability (i.e., 1 pg/ml).

Pheromone Collection Based on the behavioral results, and in addition to the week zero newly emerged females (N=8) euthanized at the beginning of the study, we also euthanized subsets of the week two newly emerged females (N=8) and 2 wk post-emergence females (N=8). Snakes were euthanized via an overdose of Brevital sodium (methohexital), then placed individually in glass beakers (500-ml), covered with 100 ml of 100% hexane (C_6H_{12}), and allowed to soak overnight (Mason et al., 1989, 1990). Excess solvent was eliminated from the resulting skin lipid extracts under reduced pressure by rotoevaporation at 35°C. The remaining residues were weighed on a digital scale (Ohaus Adventurer Pro AV 264), resuspended in 5 ml of fresh hexane, and stored at −20°C in 9-ml glass vials with polyethylene lined caps.

In order to isolate the methyl ketones that comprise the sexual attractiveness pheromone, skin lipid extracts were fractionated using column chromatography (as described by Mason et al., 1989). In brief, skin lipid extracts were loaded onto glass columns (11 mm ID) packed with alumina (activity III); the columns were eluted with 30 ml of hexane and ethyl ether ($C_4H_{10}O$) solutions of increasing polarity (LeMaster and Mason, 2002). For each sample, we collected the fractions containing the appropriate methyl ketones (fractions 5 and 6); excess solvent was removed by rotoevaporation (35°C). The resulting residues were weighed on a digital scale, resuspended in 2 ml of fresh hexane, and stored at −20°C in 9-ml glass vials with polyethylene lined caps until further analysis.

Pheromone Analysis To examine qualitative variation in pheromone expression, we determined the number and relative concentrations of structurally unique methyl ketones expressed by each female. The methyl ketones were identified using a Hewlett Packard 5890 series II gas chromatograph fitted with a split injector (280°C) and a Hewlett Packard 5971 mass selective detector. Aliquots (1 μl) of the methyl ketone fractions were injected onto a fused-silica capillary column (HP-1; 12 m×0.22 mm ID; Hewlett Packard); helium was used as the carrier gas. Oven temperature was initially held at 70°C for 1 min, then increased by 30°C/min to 210°C, where it was held for 1 min, then increased by 5°C/min to 310°C, where it was held for 5 min. After identifying the methyl ketones, we used peak integration to calculate the relative concentrations of individual methyl ketones in each sample. Compounds and peak areas were identified using ChemStation software (Version B.02.05; Hewlett Packard) interfaced with the gas chromatograph–mass spectrometer. Variations in pheromone quantity were examined by calculating the amount of methyl ketones (to the nearest μg/ml) expressed per unit skin surface area (μg/cm^2) for each female. Multiplication of snout–vent length by mid-body circumference was used to determine a general index of skin surface area for each female (Mason et al., 1990). To determine the concentration of methyl ketones, an external standard of known concentration (methyl stearate–10 μg/ml; 0.5 μl aliquot) was injected into the GC/MS with each sample.

Statistical Analysis Statistical analyses were performed using Jandel SigmaStat software (version 3.11, Systat Software, Inc.) and R (v.1.8-8). Student's *t*-tests were used to compare female SVL and mass among treatment groups in the behavioral trials. Differences in the proportions of males courting females in the various treatments were examined using *Chi-square* analyses (Zar, 1999). As the propensity for male courtship behavior changes over the course of the breeding season (O'Donnell et al., 2004), courtship only was compared within specific time points. Estradiol levels, after being log-transformed to correct for non-normality, were compared using one-way analysis of variance (ANOVA) with Tukey's *post hoc* tests used to make pairwise comparisons.

The SVL and mass of females used in pheromone analyses were compared between treatments with one-way ANOVA, which was also used to examine differences in the amounts of skin lipids and methyl ketones expressed by females. Fisher's exact tests were used to compare differences in the proportions of females expressing each individual methyl ketone with Tukey-type *post hoc* tests used to make necessary pairwise comparisons (Zar, 1999). Differences in the relative concentrations of individual methyl ketones were analyzed using the Multi-Response Permutation Procedure (MRPP) in the vegan package for R with pairwise comparisons carried out by the same procedure but

with a new group excluded each time (McCune et al., 2002; Parker and Mason, 2009). MRPP is a non-parametric multivariate analysis used to detect differences among groups (McCune et al., 2002; Mielke and Berry, 2007). Coordinates for a non-metric multidimensional scaling plot to visually represent differences in individual pheromone profiles were also generated with the vegan package. All graphics were created in SigmaPlot (version 9.01; Systat Software, Inc.).

Results

Behavioral Experiments Snout-vent length (SVL) and mass did not differ significantly between week one newly emerged females and one week post-emergence females (Student's *t*-tests; SVL: $t=-1.042$, $P=0.318$; mass: $t=1.414$, $P=0.183$) or between week two newly emerged females and two week post-emergence females (Student's *t*-tests; SVL: $t=-0.384$, $P=0.706$; mass: $t=1.065$, $P=0.302$). Two week post-emergence females were courted by a lower proportion of males than were week two newly emerged females tested on the same day ($\chi^2=4.686$, *d.f.* = 1, $P=0.030$, Fig. 1). There was no difference in the proportion of males courting 1 wk post-emergence females compared to week one newly emerged females ($\chi^2<0.001$, *d.f.* = 1, $P=1.000$, Fig. 1).

Hormone Analysis Estradiol concentrations varied significantly between treatments (one-way ANOVA, $F=4.532$, $P=0.022$, Fig. 2). Pairwise comparisons (Tukey's tests) indicated that estradiol concentrations were lower in 2 wk post-emergence females compared to week zero newly emerged females ($P=0.017$). Despite similar estradiol concentrations between 1 wk and 2 wk post-emergence females ($P=0.328$), the estradiol concentrations of 1 wk post-emergence females did not differ from those of week zero newly emerged females ($P=0.378$). This lack of a statistically significant difference may be due to relatively low sample sizes ($N=8$, 7, and 10 for zero week newly emerged, 1 wk post-emergence, and 2 wk post-emergence females, respectively) and consequent low power.

Pheromone Quantity Neither SVL nor mass differed between the week zero newly emerged females, week two newly emerged females, and 2 wk post-emergence females from which pheromone samples were collected (one-way ANOVA; SVL: $F=2.671$, $P=0.093$; mass: $F=0.968$, $P=0.396$). The total amounts of skin lipids (mg) extracted from females did not vary significantly between treatments (one-way ANOVA, $F=3.021$, $P=0.070$, Table 1). Once variation in skin surface area was taken into account, the average concentration ($\mu g/cm^2$) of methyl ketones expressed on the skin surface of females also did not differ between treatments (one-way ANOVA, $F=1.723$, $P=0.203$, Table 1).

Pheromone Quality Following GC/MS analysis of the methyl ketone fractions, eighteen structurally unique long-chain methyl ketones were identified across the samples. Of these, nine were identified as long-chain saturated methyl ketones, ranging in size from 394 to 506 daltons; the remaining nine were identified as long-chain ω-9 *cis*-unsaturated methyl ketones, ranging in size from 420 to 532 daltons (Fig. 3). For sixteen of the methyl ketones, the proportion of females expressing a particular compound did not differ between treatments (Fisher's exact tests, $P>0.05$). Two compounds, 420 dalton and 434 dalton methyl ketones, were expressed by a

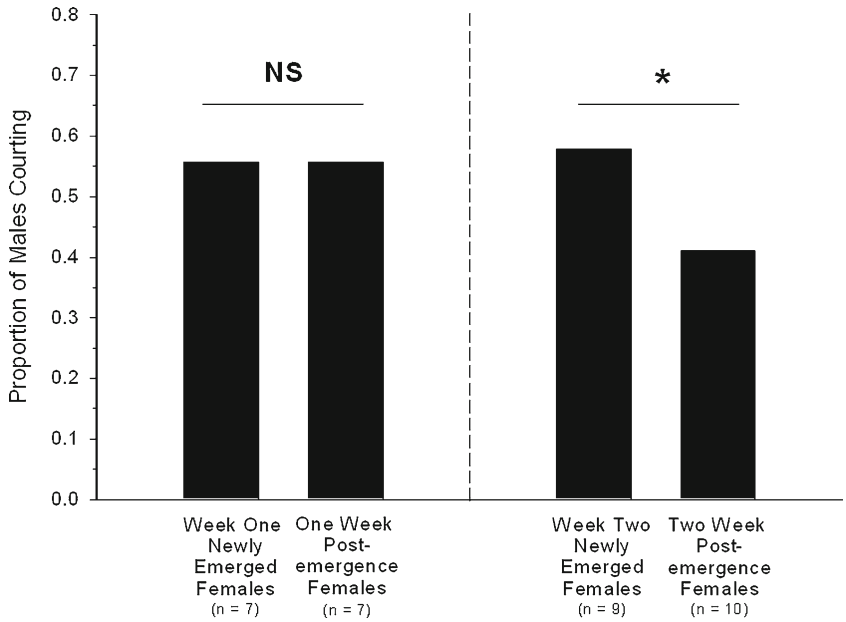

Fig. 1 Total proportion of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) courting females at two time points during the breeding season. Females were newly emerged from hibernation or were post-emergence females having been out of hibernation for either one or two weeks. * $P<0.05$

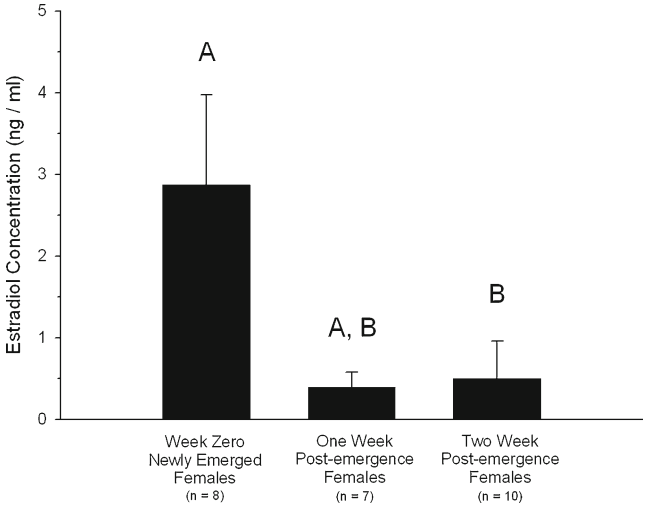

Fig. 2 Mean (+ SE) plasma estradiol concentrations of female red-sided garter snakes (*Thamnophis sirtalis parietalis*) newly emerged from hibernation, 1 wk post-emergence, and 2 wk post-emergence. Bars followed by different letters are statistically significant at $P<0.05$

greater proportion of females in the 2 wk post-emergence treatment (Tukey-type pairwise comparisons, $P<0.001$).

In terms of the relative concentrations of individual methyl ketones, pheromone profiles varied between treatments (MRPP, $A=0.118$, $P=0.002$, Fig. 4a). Week zero newly emerged females and week two newly emerged females did not differ from one another (MRPP, $A=-0.035$, $P=0.895$) although each newly emerged group was different from the two week post-emergence females (MRPP; $A=0.167$, $P<0.001$ and $A=0.149$, $P<0.001$, respectively). When plotted on a non-metric multi-dimensional scaling plot, the pheromone profiles of 2 wk post-emergence females clustered together and were distinct from the profiles of newly emerged females (Fig. 4b).

Discussion

In accordance with previous work by Shine et al. (2005a), the results of our behavioral trials indicate the attractivity of female red-sided garter snakes declines following emergence from hibernation. Subsequent chemical analyses revealed that the pheromone profiles of newly emerged females were significantly different from those of females that were two weeks post-emergence. Specifically, we observed differences in the proportions of females expressing certain methyl ketones as well as changes in the relative concentrations of individual methyl ketones. These results indicate that the female sexual attractiveness pheromone exhibits variation following emergence that may account for the intraseasonal decline in attractivity. Furthermore, estradiol levels were significantly lower in two week post-emergence females than in week zero newly emerged females, supporting the hypothesis that estrogen has a role in mediating the change in pheromone expression.

Based on the current and previous studies, it is clear that the attractivity of female red-sided garter snakes declines intraseasonally. However, the biological significance of the decline has yet to be thoroughly investigated despite the proposals of interesting hypotheses. Shine et al. (2005b), for example, suggested that a decline in female attractivity following emergence could benefit female fitness by limiting the amount of time they are subject to intensive courtship. The vigorous courtship characteristic of this species can be costly for females as it increases the risk of mortality (Shine et al., 2001, 2003b, 2004) and impedes dispersal to the summer feeding grounds, reducing the time females are able to feed (Shine et al., 2005b). Alternatively, declining female attractivity could be beneficial to male fitness: by directing courtship toward newly emerged females, which are relatively weak and less able to resist copulation attempts, males increase their chances of successfully mating (Shine et al., 2005a).

The decline in female attractivity observed in the current study occurred independently of changes in male behavior. Two week post-emergence females elicited less courtship than did week two newly emerged females tested on the same day, indicating that a post-emergence change occurred with respect to females. Male courtship behavior did not differ between week one newly emerged females and one week post-emergence females; however, this may be a reflection of testing conditions. As males in our study were presented with only a single female, it is possible that the attractivity of one week post-emergence females had not declined greatly enough for males to forgo the opportunity

Table 1 Snout-vent length, mass, total skin lipids, and overall methyl ketone concentration (per unit skin surface) for female red-sided garter snakes (*Thamnophis sirtalis parietalis*) in three treatment groups from which pheromone samples were collected. Values represent the mean ± SE

Female treatment group	Snout-vent length (cm)	Mass (g)	Skin lipids (mg)	Methyl ketones (μg/cm^2)
Week zero newly emerged ($N=8$)	64.1±0.4	94.0±3.6	19.38±1.66	1.53±0.35
Week two newly emerged ($N=8$)	62.9±0.7	87.3±3.5	19.50±1.20	2.88±0.96
Two week post-emergence ($N=8$)	62.7±0.3	90.1±3.2	24.75±2.27	3.24±0.61
Statistical analysis (ANOVA)	$F=2.671$, $P=0.093$	$F=0.968$, $P=0.396$	$F=3.021$, $P=0.070$	$F=1.723$, $P=0.203$

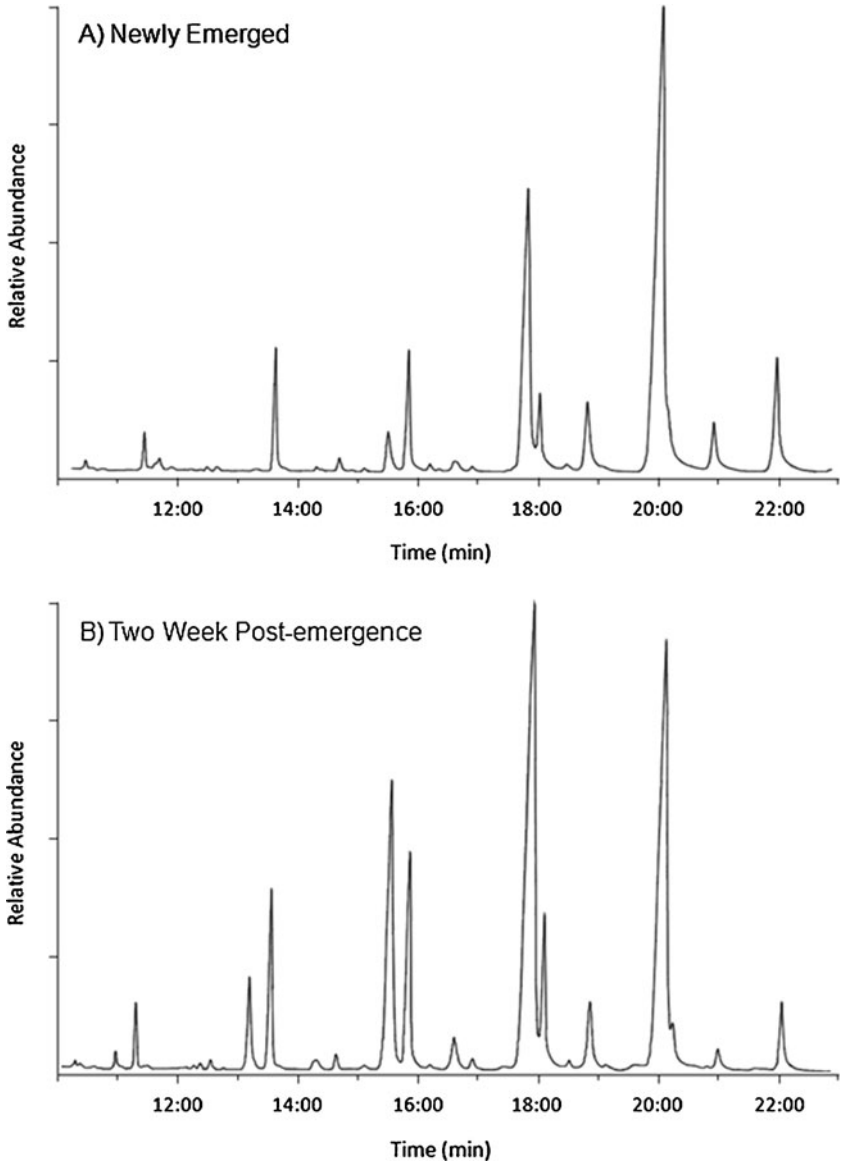

Fig. 3 Chromatograms depicting representative pheromone profiles of **a**) zero week newly emerged and **b**) 2 wk post-emergence female red-sided garter snakes (*Thamnophis sirtalis parietalis*)

to mate when no alternative female was present. Also of note is that we are describing an intraseasonal decline in attractivity distinct from that which occurs in female garter snakes after mating. The post-mating loss of attractivity results from the male's deposition of a copulatory pheromone, which reduces male courtship (Whittier et al., 1985; Shine et al., 2000b; Mendonça and Crews, 2001). In the current study, mating was prevented during behavioral trials in order to avoid the confounding effects of this mating-induced loss of attractivity.

Since the chemical identification of the female sexual attractiveness pheromone, a number of studies have demonstrated the importance of methyl ketones in garter snake reproduction (Mason et al., 1989, 1990; LeMaster et al., 2001; LeMaster and Mason, 2001b, 2002, 2003; Parker, 2010). Males are not only able to discriminate among females with qualitatively different methyl ketone profiles (LeMaster and Mason, 2002, 2003), but also can discern differences among methyl ketone profiles presented as isolated samples devoid of other female cues (Mason et al., 1989, 1990; LeMaster and Mason, 2002). Similar to previous studies that demonstrated that methyl ketone profiles vary according to species, population, season, and body size (LeMaster and Mason, 2001a, 2002, 2003; Mason and Parker, 2010), our results show variation in the relative concentrations of individual methyl ketones between treatments. In addition, we found that two methyl ketones (420 and 434 daltons, respectively) were expressed by a greater proportion of two week post-emergence females. This was somewhat unexpected as variation in the proportion of females expressing particular methyl ketones has not been documented previously.

As both methyl ketones differentially observed in post-emergence females were compounds present in low

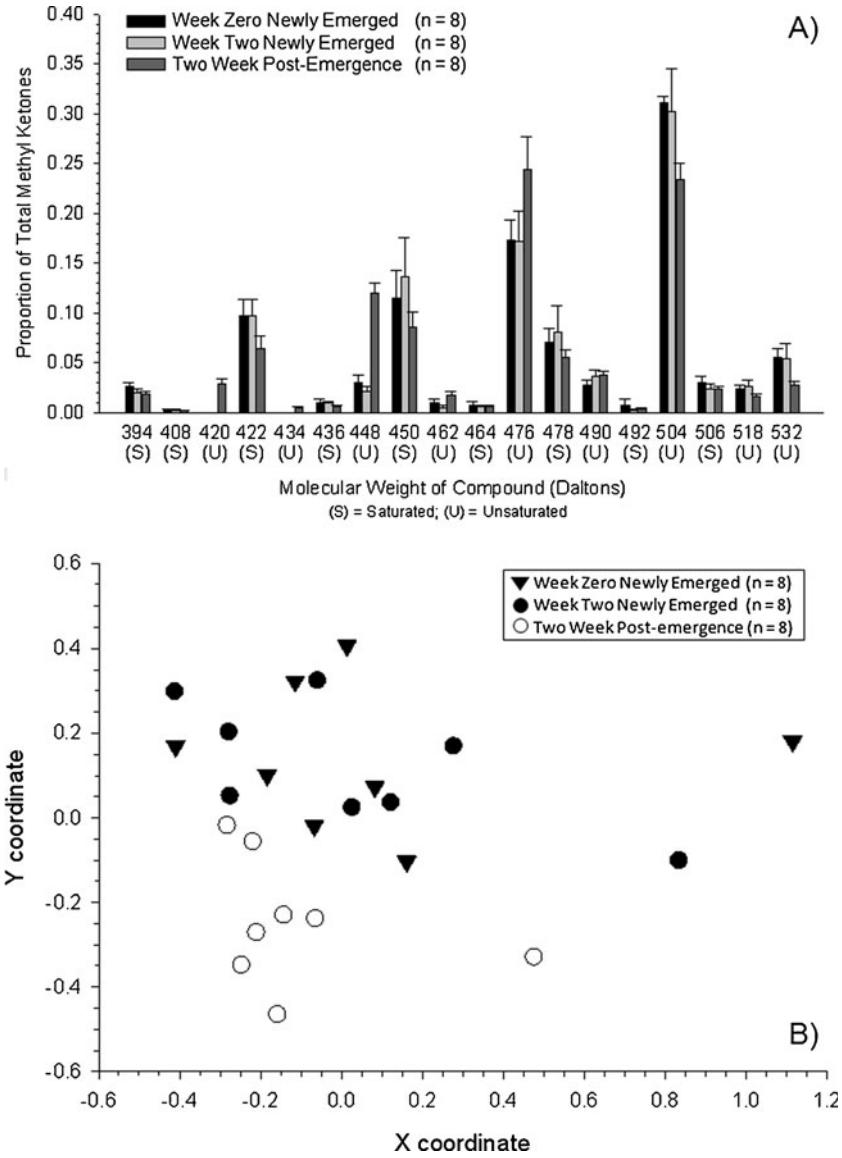

Fig. 4 **a** Relative proportions of individual methyl ketones expressed in the skin lipids of female red-sided garter snakes (*Thamnophis sirtalis parietalis*) newly emerged from hibernation and females 2 wk post-emergence. Bars represent mean + SE. **b** Non-metric multidimensional scaling plot of individual pheromone profiles for newly emerged and 2 wk post-emergence females. Each point represents the pheromone profile of an individual female

concentrations in garter snake skin lipids (LeMaster and Mason, 2002, 2003), it is possible that they were also present in newly emerged females, but at levels below our threshold of detectability. Indeed, although the difference was not significant, post-emergence females had a higher overall concentration of methyl ketones in their skin lipids compared to newly emerged females. Such an upregulation of lipid-based compounds is not surprising considering that, in addition to serving as semiochemicals, skin lipids also play a role in reducing transcutaneous water loss (Lillywhite and Maderson, 1982; Burken et al., 1985; Mason et al., 1987), a function particularly relevant to garter snakes upon emergence from hibernation.

Whether newly synthesized or simply upregulated, the 420 and 434 dalton methyl ketones may indicate compounds that have inhibitory effects on male courtship behavior similar to, but less transitory than, the effect of the aforementioned copulatory pheromone. Alternatively, it is possible that these methyl ketones have little or no role in the loss of female attractivity, which may instead be mediated by other changes in the relative concentrations of individual methyl ketones. As the exact contribution of each methyl ketone to female attractivity is not known and it was not feasible to test synthetic methyl ketones in the current study, the precise nature of the chemical variation that may be involved in the loss of female attractivity is unclear. However, our overall finding that methyl ketone profiles vary between newly emerged females and post-emergence females, which correlates with the results of behavioral trials, is evidence that the intraseasonal decline in female attractivity is chemically mediated. Despit this evidence, we recognize that future studies presenting male snakes with synthetic methyl ketones will be necessary to demonstrate

definitively the role of the pheromone in mediating intraseasonal declines in female attractivity.

The physiological mechanisms that underlie the intraseasonal variation in the sexual attractiveness pheromone remain unclear although the results of our hormone analysis suggest the involvement of estrogen. Two week post-emergence females had lower estradiol than did newly emerged females, lending support to previous studies indicating that the female sexual attractiveness pheromone of the red-sided garter snake may be estrogen regulated (Crews, 1976; Mason and Crews, 1985). Precisely how estrogen may regulate the pheromone is not known; however, prior investigations have suggested that estrogen could have a role in maintaining the pheromone in female skin lipids following emergence (Garstka and Crews, 1985; Mendonça and Crews, 1996). The results of the current study support this hypothesis: the decline in female attractivity and the pheromonal changes were accompanied by a decrease in estrogen, as would be expected if indeed estrogen is responsible for pheromone maintenance.

Instead of, or perhaps in addition to, being hormonally regulated, intraseasonal variation in the female sexual attractiveness pheromone may be impacted by environmental conditions. This seems particularly likely given that pheromone profiles did not differ between week zero newly emerged females and week two newly emerged females indicating that changes occur only after females have emerged from the hibernaculum. Indeed, changes in environmental conditions have been shown to affect the cuticular hydrocarbon profile of the harvester ant (*Pogonomyrmex barbatus*). Wagner et al. (2001) found that harvester ant workers, which spend most of their time inside the nest, developed higher proportions of saturated, unbranched hydrocarbons in their cuticle upon exposure to conditions found outside the nest (i.e., higher temperatures, lower humidity). Emerging from winter hibernation, female red-sided garter snakes experience a similar change in environment as the external conditions are warmer and drier than those of the den interior (Shine et al., 2005a). Thus, it is possible that qualitative variation in the pheromone profiles of newly emerged and two week post-emergence females is a direct reflection of the different amounts of time the snakes were exposed to environmental conditions outside the den. Further work will be required to explore this hypothesis.

Acknowledgements We thank the Manitoba Department of Conservation, Dave Roberts, and Suzanne Estes for assistance in the field and Al and Gerry Johnson for their support. We also thank Vanessa Uhrig and Chris Friesen for encouragement and help in reviewing this manuscript, Pat Aldrich for assistance with statistical methods, and two anonymous reviewers for constructive comments that helped improve this manuscript. This work was supported by a Western Oregon University Cummins Natural Sciences and Math Award to E. J.U., an NSF grant (0620125) to R.T.M., and a Western Oregon University Faculty Development Award to M.P.L.

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