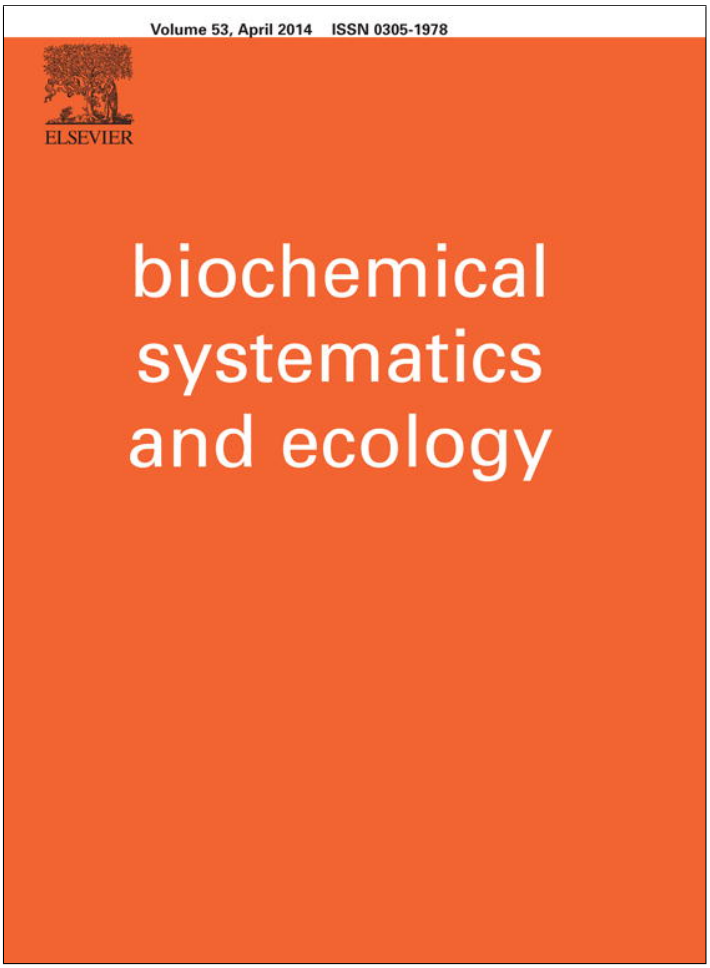

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Species specificity of methyl ketone profiles in the skin lipids of female garter snakes, genus *Thamnophis*

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ABSTRACT

One of the relatively few vertebrate pheromones to be chemically identified, the female sex pheromone of the red-sided garter snake (*Thamnophis sirtalis parietalis*) is a series of saturated and monounsaturated methyl ketones contained within female skin lipids. During the breeding season, this pheromone is responsible for eliciting male courtship behaviors and males are able to utilize pheromonal variation to discriminate among females. While the pheromone system of the red-sided garter snake has been the subject of many studies, relatively little is known about the pheromone systems of other garter snakes. Through chemical analyses, we demonstrate that female skin lipids of the red-spotted garter snake (*Thamnophis sirtalis concinnus*), northwestern garter snake (*Thamnophis ordinoides*), and plains garter snake (*Thamnophis radix*) contain similar methyl ketones. The methyl ketone profiles of these snakes differ qualitatively from one another and from the methyl ketone profiles of red-sided garter snakes with differences particularly pronounced between sympatric species. Our results provide evidence that the use of methyl ketones in sexual signaling may be ubiquitous for *Thamnophis* species and suggest that these compounds could play a role in reproductive isolation between species in this genus.

1. Introduction

Sexual signals convey information about attributes of the signaler such as species, sex, health, and/or reproductive status (West-Eberhard, 1983; Ryan and Rand, 1993; von Schantz et al., 1999; Clutton-Brock, 2007). This information can then be used by the signal receiver to discriminate among potential mates and direct reproductive efforts toward those individuals with which copulation will be most likely to result in reproductive success. For signaling to be informative, variation must exist and, indeed, is found in numerous signaling systems and across a range of sensory modalities (Endler, 1992; Ryan and Rand, 1993; Penn and Potts, 1998; von Schantz et al., 1999). When variation in sexual signaling occurs between similar species it can have a role in maintaining reproductive isolation at the pre-zygotic level. That is, the signal receiver is able to discriminate between conspecifics and heterospecifics based on the signal and thus avoid mating with a potentially incompatible heterospecific individual with which offspring may be inviable or have otherwise reduced fitness (Ryan and Rand, 1993; Pfennig, 1998; Burke and Arnold, 2001). This variation is particularly relevant for sympatric species that have temporally overlapping breeding seasons.

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Pheromones, by definition a species specific form of chemical communication (Karlson and Lüscher, 1959), are used in sexual signaling and mate choice by numerous organisms (Wyatt, 2003). Chemical variation distinguishing the pheromones of different species, such as variation in the structures or relative ratios of compounds, has most often been described in insects [e.g. arctiid moths (*Creatonotos* sp.; Bell and Meinwald, 1986); fruit flies (*Drosophila* sp.; Ferveur, 2005); ants (*Formica* sp.; Martin et al., 2008)] although it is likely relevant for species recognition in many vertebrates as well. Certain fish (e.g. *Xiphophorus* sp.; Wong et al., 2005), lizards (e.g. *Podarcis* sp.; Barbosa et al., 2006), and rodents (e.g. *Apodemus* sp.; Simeonovska-Nikolova, 2007) have been shown to discriminate between conspecifics and heterospecifics in behavioral trials based on chemical cues alone. However, few studies of vertebrates include chemical analyses and thus, the precise nature of the chemical variation underlying the discrimination remains unknown in most cases [but see Shine et al., 2002 – (Laticaudine sea snakes) and Iwata et al., 1999 (*Cynops* newts) for notable exceptions].

In garter snakes of the genus *Thamnophis*, the dorsal skin lipids of females contain a sexual attractiveness pheromone that elicits male courtship behaviors during the breeding season (Mason et al., 1990; Mason, 1992, 1993). For *Thamnophis sirtalis parietalis* (red-sided garter snake), the pheromone has been chemically identified as a homologous series of eighteen long chain saturated and monounsaturated methyl ketones (Mason et al., 1989, 1990; Mason, 1992, 1993; LeMaster and Mason, 2002). Pheromone profiles exhibit temporal variation as well as both inter- and intrapopulational variation which can be used by males to discriminate among females (LeMaster and Mason, 2001, 2002, 2003; Uhrig et al., 2012). Behavioral studies have provided evidence that male garter snakes can also discriminate con- and heterospecific pheromone trails (Ford, 1982; Ford and Schofield, 1984). However, prior to the current study, pheromone profiles of *Thamnophis* species or subspecies other than *T. s. parietalis* have not been chemically analyzed.

In this study, we compare the methyl ketone profiles found in female skin lipids of *Thamnophis radix* (plains garter snakes), *Thamnophis ordinoides* (northwestern garter snakes), *Thamnophis sirtalis concinnus* (red-spotted garter snakes), and *T .s. parietalis*, the latter two of which are subspecies of the common garter snake. *T. radix* is sympatric with *T. s .parietalis* at the northern extent of its range in Manitoba, Canada while *T. ordinoides* and *T. s .concinnus* are sympatric in the western United States and allopatric to both *T. radix* and *T. s. parietalis* (Rossman et al., 1996). Based on previous studies of *T. s. parietalis* pheromonal variation (e.g. LeMaster and Mason, 2001, 2002, 2003), we would expect chemical profiles to vary in terms of methyl ketone quantity and/or quality (e.g. relative concentrations of compounds) between species. As sympatric species are likely to encounter one another during their temporally overlapping breeding seasons, there may be selective pressure for the divergence of profiles via reinforcement (Coyne and Orr, 1989; Noor, 1999); thus we would expect the greatest variation between the profiles of sympatric species. The two common garter snake subspecies may have similar profiles due to their phylogenetic relatedness; however, similarity in pheromone profiles does not always predictably follow phylogenetic patterns (Symonds and Elgar, 2004; Ferveur, 2005).

2. Materials and methods

2.1. Study species and collection

Adult female *T. s. concinnus* (red-spotted garter snakes, $N = 13$) and *T. ordinoides* (northwestern garter snakes, $N = 10$) were collected during early April at the E.E. Wilson Wildlife Area north of Corvallis, Oregon (44°30'N, 123°17'W). Adult female *T. s. parietalis* (red-sided garter snakes, $N = 10$) and *T. radix* (plains garter snakes, $N = 8$) were collected during late April in the Interlake region of Manitoba, Canada near the towns of Inwood (50°37′N, 97°32′W) and St. Laurent (50°24′N, 97°56′W), respectively. Collection periods coincided with the breeding seasons of these snakes and all snakes were hand captured. For *T. s. parietalis*, which forms large breeding aggregations as they emerge en masse from winter hibernation ($\geq$35,000 snakes per hibernacula; Shine et al., 2006), females were indiscriminately collected immediately upon emergence to ensure their status as unmated seasonal virgins. The same collection method could not be used for *T. s. concinnus*, *T. ordinoides*, and *T. radix*, as these snakes are not known to form such large aggregations and are much more dispersed during the breeding season. Thus, females of these species were opportunistically collected as they were encountered in the field and their cloacae inspected for the presence of copulatory plugs which would indicate a recent mating (Shine et al., 2000). Only females from which copulatory plugs were absent were used for pheromone collection.

2.2. Pheromone collection

As pheromone profiles have been demonstrated to vary with body size (LeMaster and Mason, 2002), all snakes were weighed and measured to ensure there was no size bias between groups. Pheromone samples were obtained by euthanizing females (*T. s. parietalis*: $n = 10$; *T. radix*: $n = 8$; *T. s. concinnus*: $n = 13$; *T. ordinoides*: $n = 10$) with an overdose of Brevital® sodium (methohexital). Following euthanasia, snakes were placed individually in glass beakers, covered with 100% hexane (C_6H_{14}), and soaked overnight to extract skin lipids (Mason et al., 1989, 1990). The resulting extracts were transferred to glass vials and transported to Western Oregon University where rotoevaporation (35 °C) was used to remove excess solvent. Lipid residues were weighed to the nearest milligram on a digital scale (Ohaus Adventurer Pro AV 264), resuspended in 5 ml of fresh hexane, and stored at −20 °C in glass vials until fractionation could be conducted.

To isolate methyl ketones, the skin lipid extracts were fractionated via column chromatography as described by Mason et al. (1989). Briefly, skin lipid extracts were loaded onto glass columns (11 mm ID) packed with alumina (Activity III).

The columns were then eluted with a series of increasingly polar hexane and ethyl ether ($C_4H_{10}O$) solutions (LeMaster and Mason, 2002). For each sample, the fractions containing the methyl ketones (fractions 5 and 6) were collected and excess solvent was again removed by rotoevaporation. The resulting residues were weighed, resuspended in 2 ml of fresh hexane, and stored at −20 °C in glass vials until further analysis.

2.3. Pheromone analysis

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 containing fractions were injected onto a fused-silica capillary column (HP-1; 12 m × 0.22 mm ID; Hewlett Packard) with helium used as the carrier gas. An internal standard of known concentration (methyl stearate – 10 mg/ml; 0.5 μl aliquot per sample) was injected with each sample. 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. Compound identities and peak areas were determined using ChemStation software (Version B.02.05; Hewlett Packard) interfaced with the gas chromatograph–mass spectrometer. After identifying the compounds, peak integration was used to calculate the concentrations of individual methyl ketones in each sample by comparing the area of each methyl ketone peak to that of the methyl stearate standard. An index of skin surface area was determined for each female by multiplication of snout-vent length (SVL) and mid-body circumference (Mason et al., 1990); this was then used to calculate the amount of methyl ketones expressed per unit skin surface ($\mu g/cm^2$).

2.4. Statistical analyses

Mass, SVL, and total skin lipid quantity were compared among species and subspecies using one way analysis of variance (ANOVA) with *post-hoc* Tukey tests used to make pairwise multiple comparisons where appropriate. Due to unequal variance the percentage of skin lipids comprised of methyl ketones, methyl ketone concentrations per unit skin surface, and the percentage of total methyl ketones that were unsaturated compounds were compared among groups using non-parametric Kruskall–Wallis one-way ANOVA on ranks with *post-hoc* Dunn's tests used for pairwise multiple comparisons. The proportion of females in each group expressing a particular methyl ketone compound was compared using Fisher's exact tests with Tukey-type *post-hoc* multiple comparisons (Zar, 1999). Multi-response permutation procedures [MRPP from the vegan package in R (Oksanen et al., 2010)] were used to evaluate interspecific differences in the relative concentrations of individual methyl ketones with pairwise comparisons performed using the same procedure but excluding different groups each time (Parker and Mason, 2009; Uhrig et al., 2012). Coordinates for a non-metric multidimensional scaling plot were also generated in the vegan package and used to create a graphical representation of differences in pheromone profiles. All statistical analyses were performed with Jandel SigmaStat software (version 3.11, Systat Software, Inc.) and R (v.1.8–8; R Development Core Team 2009). All graphics were created in SigmaPlot (version 9.01; Systat Software, Inc.).

3. Results

There were no differences in snout-vent length (SVL) or mass among species or subspecies (one-way ANOVA; mass: $F = 0.016$, $P = 0.997$; SVL: $F = 0.245$, $P = 0.864$). The overall amount of total skin lipids also did not differ among groups (one-way ANOVA, $F = 2.415$, $P = 0.082$). The percentage of skin lipids comprised of methyl ketones was significantly lower for *T. s. parietalis* compared to the other groups [*post-hoc* Dunn's pairwise comparisons ($P < 0.05$) following Kruskall Wallis one-way ANOVA on ranks, $H = 27.497$, $P < 0.001$; Table 1]. Similarly, *T. s. parietalis* expressed a lower concentration of methyl ketones per unit skin surface [*post-hoc* Dunn's pairwise comparisons ($P < 0.05$) following Kruskall Wallis one-way ANOVA on ranks, $H = 22.272$, $P < 0.001$; Table 1].

A total of eighteen unique methyl ketones ranging in molecular weight from 394 to 532 Da were identified across the samples (Fig. 1, Table 2). Within individual females, methyl ketone expression ranged from nine compounds to the full suite of

Table 1
Mean (±SE) and median values (with quartiles) for mass, snout-vent length (SVL), total skin lipids, percentage of total skin lipids comprised of methyl ketones, concentration of methyl ketones per unit skin surface, and percentage of methyl ketones that are unsaturated compounds for female garter snakes.

Species	Mass (g)	SVL (cm)	Total skin lipids (mg)	Methyl ketones (% total skin lipids)	Methyl ketones ($\mu g/cm^2$)	Unsaturated methyl ketones (% total methyl ketones)
Thamnophis sirtalis parietalis ($N = 10$)	52.6 ± 5.8	51.9 ± 2.2	16.2 ± 2.5	0.59 ± 0.12	0.55 ± 0.15	0.32 ± 0.07
	53.2 (38.2; 69.3)	51.9 (47.4; 57.3)	16.0 (10.0; 19.3)	0.55 (0.31; 0.86)	0.45 (0.25; 0.80)	0.22 (0.17; 0.53)
Thamnophis radix ($N = 8$)	51.8 ± 10.8	48.9 ± 3.1	16.3 ± 2.3	4.67 ± 0.89	4.28 ± 0.92	0.83 ± 0.03
	40.5 (27.9; 81.9)	45.7 (42.4; 56.3)	14.5 (11.3; 23.5)	4.20 (2.22; 7.41)	3.90 (1.68; 6.73)	0.85 (0.74; 0.88)
Thamnophis sirtalis concinnus ($N = 13$)	53.0 ± 5.5	50.6 ± 1.8	22.6 ± 2.3	3.10 ± 0.25	3.62 ± 0.39	0.80 ± 0.02
	51.4 (41.1; 63.5)	52.2 (45.5; 54.5)	23.0 (14.5; 29.0)	2.95 (2.36; 3.81)	3.20 (2.30; 5.15)	0.81 (0.76; 0.85)
Thamnophis ordinoides ($N = 10$)	51.0 ± 8.1	50.4 ± 2.6	16.1 ± 1.5	5.17 ± 0.44	4.53 ± 0.55	0.87 ± 0.01
	46.6 (34.1; 67.4)	49.8 (46.8; 57.0)	16.5 (12.5; 19.8)	4.91 (4.08; 6.16)	4.65 (3.05; 5.28)	0.86 (0.85; 0.90)

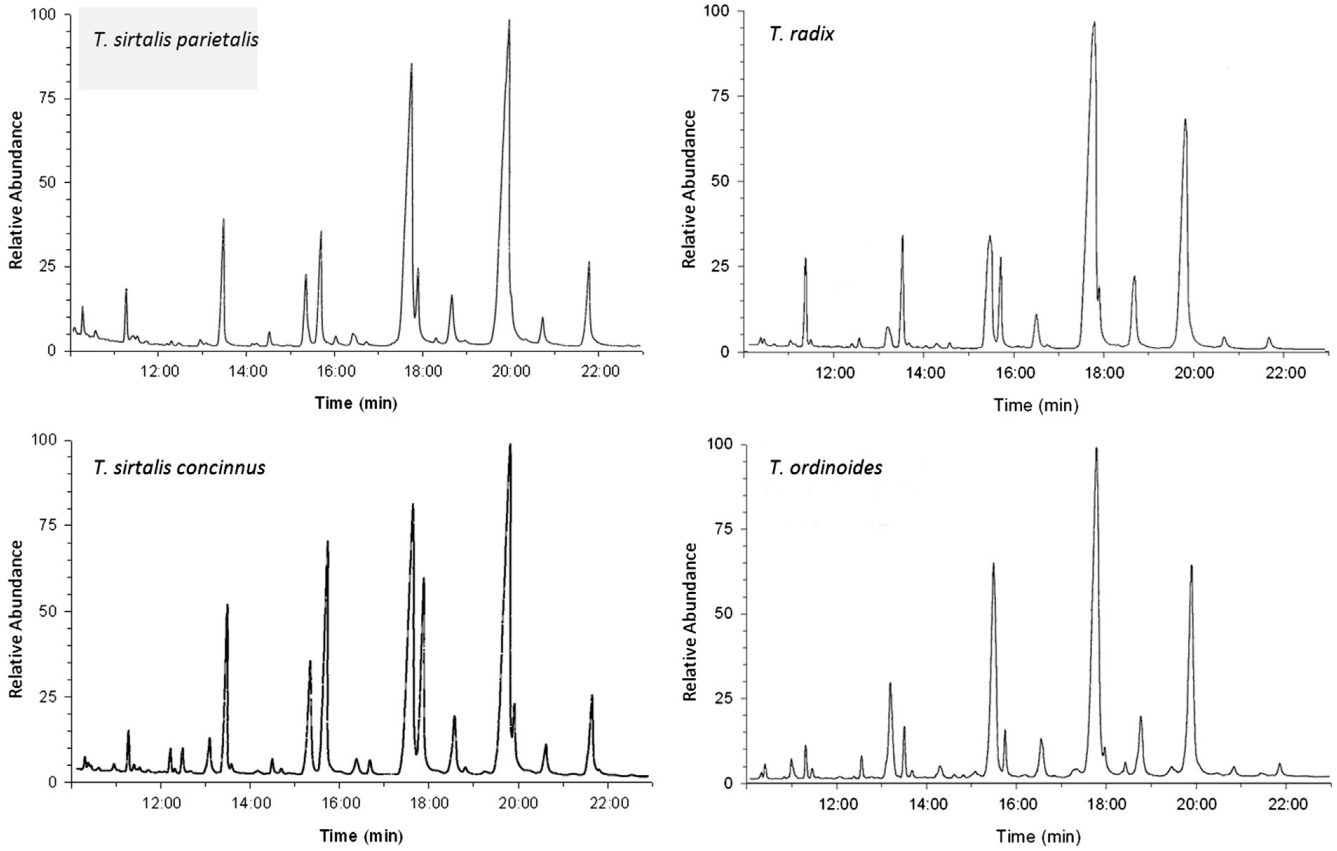

Fig. 1. Gas chromatograms depicting representative methyl ketone profiles isolated from the skin lipids of female garter snakes (*T. s. parietalis*, *T. radix*, *T. s. concinnus*, and *T. ordinoides*).

eighteen. With the exception of the 492 Da methyl ketone, which was not expressed by any of the *T. radix* females, all methyl ketones appeared in the profile of at least one individual female in each group (Table 2). The proportion of females expressing a particular methyl ketone varied between groups for six of the compounds (420, 434, 462, 506, 518, 532 Da; Fisher's exact tests followed by Tukey-type multiple pairwise comparisons; $P < 0.050$ for each; Table 2); the other twelve compounds were not differentially expressed by species or subspecies (Fisher's exact tests; $P > 0.050$; Table 2).

Multi-response permutation procedures indicated that the relative concentrations of individual methyl ketones differed significantly among groups (MRPP; $A = 0.453$, $P < 0.001$). Subsequent pairwise comparisons revealed all species and subspecies to differ significantly from one another in terms of the relative concentrations of individual methyl ketones (MRPP; *T. s. parietalis* vs. *T. radix*: $A = 0.412$, $P < 0.001$, *T. s. parietalis* vs. *T. s. concinnus*: $A = 0.409$, $P < 0.001$, *T. s. parietalis* vs. *T. ordinoides*: $A = 0.478$, $P < 0.001$, *T. s. concinnus* vs. *T. radix*: $A = 0.148$, $P < 0.001$, *T. s. concinnus* vs. *T. ordinoides*: $A = 0.279$, $P < 0.001$, *T. radix* vs. *T. ordinoides*: $A = 0.138$, $P = 0.002$; Fig. 2). Additionally, for female *T. s. parietalis* unsaturated compounds made up a lower proportion of their total methyl ketones compared to all other species and subspecies [Kruskal Wallis one-way ANOVA on ranks ($H = 27.497$, $P < 0.001$) followed by Dunn's post-hoc pairwise comparisons; $P < 0.050$ for all]. Plotting the methyl ketone profiles of individual females as points on a non-metric multi-dimensional scaling (NMS) plot generates distinct clusters for each species and subspecies (Fig. 3). In the case of sympatric species pairs (i.e. *T. s. parietalis*/*T. radix* and *T. s. concinnus*/*T. ordinoides*), these clusters are non-overlapping illustrating greater variation (Fig. 3). As indicated by the more

Table 2
Number of female garter snakes expressing each of the eighteen individual methyl ketone compounds identified across the samples. Asterisks are used to denote unsaturated compounds. Different letters (a–c) in a row indicate statistically different values (Fisher's exact tests followed by Tukey-type multiple comparisons, $P < 0.050$).

Species	Methyl ketone molecular weight (daltons)																	
	394	408	420*	422	434*	436	448*	450	462*	464	476*	478	490*	492	504*	506	518*	532*
Thamnophis sirtalis parietalis ($N = 10$)	10^{a}	9^{a}	2^{a}	10^{a}	1^{a}	10^{a}	8^{a}	10^{a}	4^{a}	10^{a}	10^{a}	10^{a}	8^{a}	5^{a}	10^{a}	10^{a}	6^{a}	6^{a}
Thamnophis radix ($N = 8$)	8^{a}	8^{a}	8^{b}	8^{a}	6^{b}	8^{a}	8^{a}	8^{a}	8^{b}	7^{a}	8^{a}	8^{a}	8^{a}	0^{a}	8^{a}	2^{b}	$7^{a,b}$	$8^{a,b}$
Thamnophis sirtalis concinnus ($N = 13$)	13^{a}	13^{a}	13^{b}	13^{a}	$8^{a,b}$	13^{a}	13^{a}	13^{a}	13^{b}	13^{a}	13^{a}	13^{a}	13^{a}	4^{a}	13^{a}	$11^{a,c}$	13^{b}	13^{b}
Thamnophis ordinoides ($N = 10$)	10^{a}	10^{a}	10^{b}	10^{a}	10^{b}	10^{a}	10^{a}	10^{a}	10^{b}	10^{a}	10^{a}	10^{a}	10^{a}	1^{a}	10^{a}	$4^{b,c}$	$10^{a,b}$	$10^{a,b}$

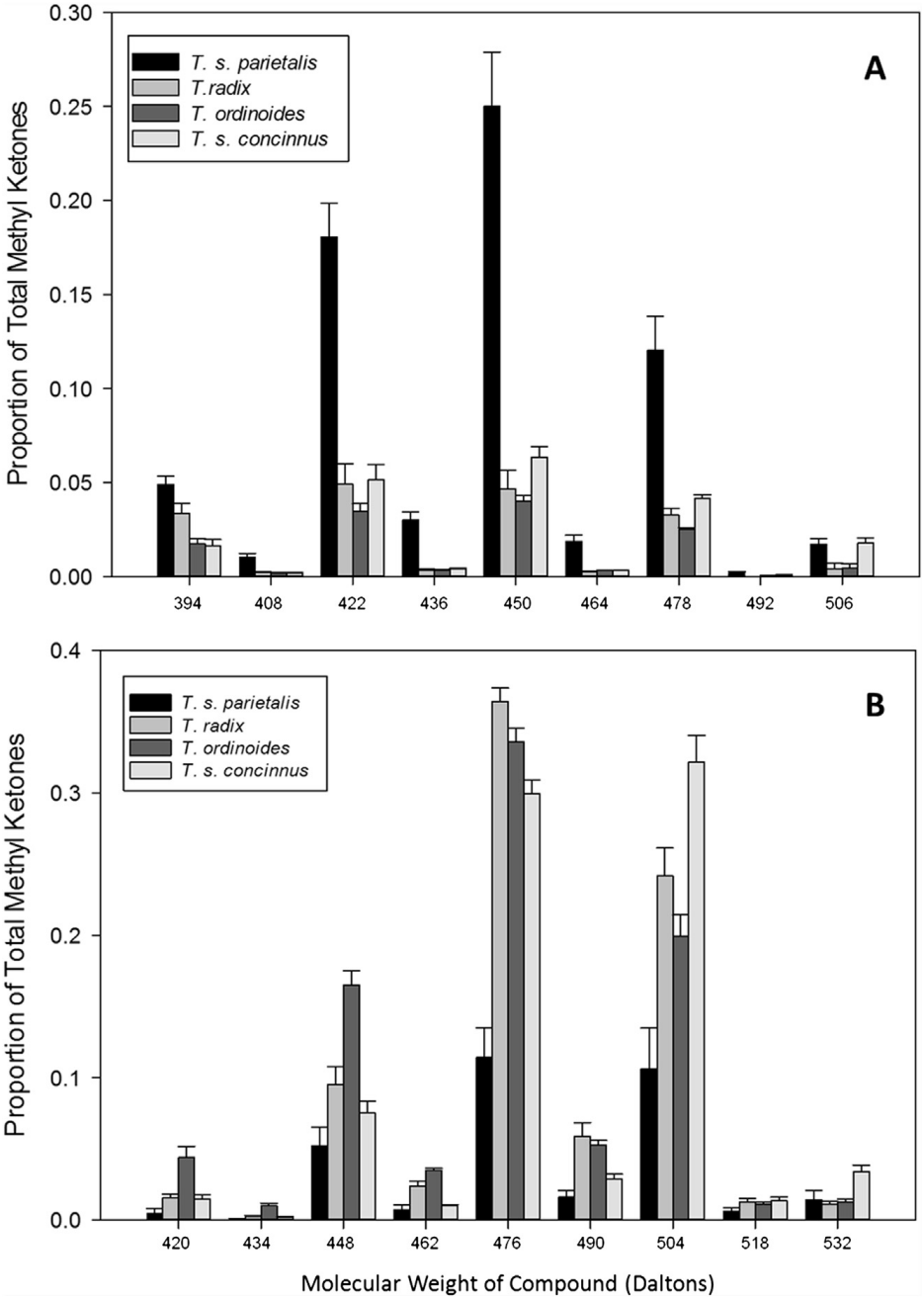

Fig. 2. Mean (±SE) relative concentrations of individual saturated (A) and unsaturated (B) methyl ketone compounds in the skin lipids of female garter snakes (*T. s. parietalis*, *T. radix*, *T. s. concinnus*, and *T. ordinoides*).

dispersed pattern of data points, the pheromone profiles of *T. s. parietalis* show relatively more within-group variation compared to the other groups. Although the group most similar to *T. s. parietalis*, as indicated by the proximity of the respective data points, is the closely related *T. s. concinnus*, these two subspecies form non-overlapping clusters on the NMS plot (Fig. 3).

4. Discussion

Our results confirm that the methyl ketone compounds previously identified in the skin lipids of *T. s. parietalis* are also present in the skin lipids of the closely related *T. s. concinnus* as well as the more distantly related *T. radix* and *T. ordinoides*. The methyl ketone profiles of all species and subspecies in this study differed from one another in terms of the relative concentrations of individual methyl ketones as well as the proportions of females expressing particular compounds. That the variation was particularly pronounced between sympatric species suggests that disruptive selection could be leading to more divergent pheromone profiles in areas of sympatry. This species specificity of methyl ketone profiles in garter snakes suggests a potential reproductive isolating role for these compounds.

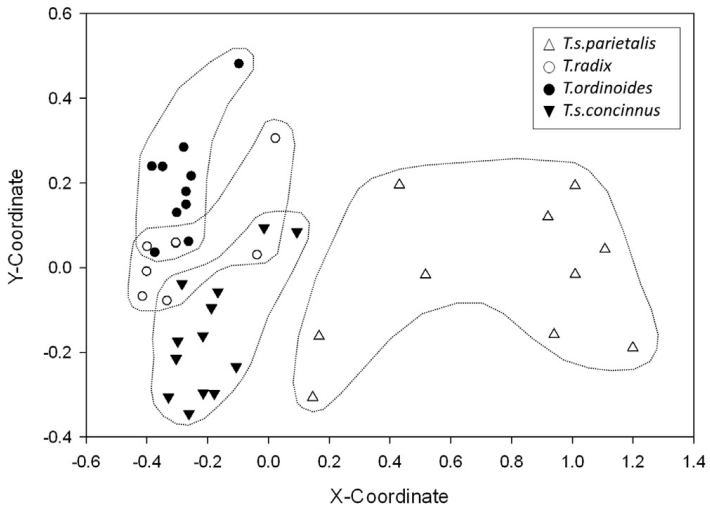

Fig. 3. Non-metric multidimensional scaling plot illustrating individual methyl ketone profiles of female garter snakes (*T. s. parietalis*, *T. radix*, *T. s. concinnus*, and *T. ordinoides*). Each data point represents the profile of an individual female snake.

In *T. s. parietalis*, methyl ketone profiles exhibit interpopulational variation as well as temporal and phenotypic variation (LeMaster and Mason, 2001, 2002, 2003; Uhrig et al., 2012). Previous studies have not compared methyl ketone profiles among different species or subspecies although behavioral experiments by Ford (1982, 1978), Ford and Schofield (1984), Ford and O'Bleness (1986) and Shine et al. (2004) using various combinations of garter snake species indicate that male snakes are able to discriminate con- and heterospecific pheromone trails and skin lipid extracts, typically preferring those of conspecifics. The aforementioned studies did not include chemical analyses; however, it was hypothesized that pheromonal variation was responsible for the observed discrimination. In our current study, the results of our chemical analyses support the previously proposed hypothesis of species specificity by demonstrating that methyl ketone profiles do indeed vary between species.

It is clear from our results that methyl ketone profiles exhibit the species specificity that would be expected if these compounds function as mechanisms of reproductive isolation. Our study complements the results of LeMaster and Mason (2003) who reported variation in methyl ketone profiles between two distinct populations of *T. s. parietalis* in Manitoba. Their study also indicated that males of at least one population are able to recognize the interpopulational variation in profiles and preferentially mate with females from their own population (LeMaster and Mason, 2003). Considering the findings of LeMaster and Mason (2003) together with our current results, it is certainly plausible that species barriers between garter snakes evolved and/or are maintained by divergence in pheromone profiles. Given that male snakes rely extensively on pheromone detection for mate choice, it is likely that pheromone variation is a particularly important component to species barriers in sympatry; however, other isolating mechanisms could be involved as well. For example, post-mating pre-zygotic mechanisms such as interspecific differences in sperm competitive ability could also play a role in the reproductive isolation of these snakes.

For many species, heterospecific matings will not produce viable offspring or will result in hybrid offspring with reduced fitness (Dobzhansky, 1936; Coyne and Orr, 1989; Arnold and Hodges, 1995; Burke and Arnold, 2001). The ability to discriminate conspecifics and heterospecifics prior to copulation could prove beneficial by increasing the likelihood of choosing a mate with which the fittest offspring can be produced. However, the fitness consequences of heterospecific matings, including the potential for hybrid inferiority, have not been studied in garter snakes. Although it is unknown whether sympatric *T. s. concinnus* and *T. ordinoides* hybridize, Shine et al. (2004) found evidence that hybridization occasionally occurs between *T. s. parietalis* and *T. radix* in Manitoba, Canada indicating that pre-zygotic isolation is not complete between these species. Behavioral experiments with *T. s. parietalis* and *T. radix* found that, while males preferred conspecific skin lipid extracts, they exhibited low intensity courtship toward heterospecific lipids as well (Shine et al., 2004). Male *T. radix* were more likely to court heterospecific lipids than were *T. s. parietalis* (Shine et al., 2004). Clearly the difference in profiles between these two species is not so great as to totally preclude the recognition of heterospecific compounds. However, this does not necessarily undermine the hypothesized role of methyl ketones as mediators of reproductive isolation. As suggested by Shine et al. (2004), it may simply be that methyl ketones are the principal of several isolating mechanisms maintaining the species barrier between *T. s. parietalis* and *T. radix*.

Based on the results of previous studies indicating that variation in the relative concentrations of compounds is important for mate choice (e.g. LeMaster and Mason, 2002, 2003), it is likely that this is the main type of variation responsible for species discrimination. However, it should be noted that we also found variation in methyl ketone quantity. Specifically, the skin lipids of female *T. s. parietalis* contained a much lower proportion of methyl ketones compared to other females although the overall amount of total skin lipids did not differ between species or subspecies. Further, the relative concentration of methyl ketones per unit skin surface was lower for *T. s. parietalis* females. The reduced methyl ketone expression of *T. s. parietalis* may,

however, be explained by our sampling methods. The *T. s. parietalis* females used in this study were all newly emerged from winter hibernation whereas the other females had likely been out of hibernation for a week or more. Lipid-based compounds play a role in reducing transcutaneous water loss and there is evidence to suggest that such compounds may be upregulated following emergence from hibernacula into the drier conditions of the den exterior (Lillywhite and Maderson, 1982; Parker and Mason, 2009; Uhrig et al., 2012). Thus, the relatively lower methyl ketone expression of *T. s. parietalis* females may simply be due to these females having had less time to upregulate these compounds following emergence.

The species in the current study, *T. sirtalis*, *T. radix*, and *T. ordinoides*, are phylogenetically distinct (Alfaro and Arnold, 2001) yet, as they all diverged from a common ancestor, it is not surprising that they all possess similar methyl ketone compounds in their skin lipids. We might expect that the degree of pheromone variation would reflect phylogenetic relatedness such that the two *T. sirtalis* subspecies would have more similar pheromone profiles. However, our results indicate that although *T. s. parietalis* profiles group closest to *T. s. concinnus* on the NMS plot, *T. s. concinnus* actually appears to be more similar to the *T. radix* than to its conspecific. Such a result is not without precedent as several studies of insects including bark beetles (*Dendroctonus* sp.; Symonds and Elgar, 2004) and fruit flies (*Drosophila* sp.; Ferveur, 2005) have found that divergence in pheromone profiles does not necessarily follow phylogenetic patterns. To the best of our knowledge, our study is the first to examine pheromones of more than two different vertebrate species in a genus. Given the number of *Thamnophis* species ($N = 30$ species recognized by Rossman et al., 1996) and their wide geographic range, future studies should examine pheromones of additional species in this genus to further elucidate the evolutionary patterns of pheromone divergence in *Thamnophis*.

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