The Role of Pheromones in the Sexual Communication of the Wolf Spider, *Schizocosa ocreata* (Araneae, Lycosidae)

DISSERTATION

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By

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Abstract

Communication between potential mates is of vital importance to the fitness of many taxa, allowing individuals to find one another, as well convey and receive information important in assessing a suitable mate. Signals involved in sexual communication can be transmitted through visual, vibrational and chemical modalities, with chemical communication being the oldest and most widespread. The ability to detect visual and vibrational cues often occurs across a range of inputs, while chemical communication has the potential for much higher specificity, as only those receivers possessing chemosensory cells attuned to the compounds used will be able to detect their presence.

The detection and integration of multimodal signals into a behavioral response has been extensively studied in the *Schizocosa ocreata* (Hentz 1844) wolf spider (Lycosidae) model system. In this ground dwelling species, males respond to substrates containing female chemical signals with visual and vibratory courtship displays. While this response has been well characterized, the chemical identity of these signaling compounds is currently unknown, and pheromone identity has been understudied across spiders as a whole. The behavioral response of male *S. ocreata* to conspecific chemical signals has primarily been studied in the context of substrate bound female cues, though past studies have suggested that chemicals produced by conspecific male competitors may inhibit male courtship behavior, and have even suggested a role for olfaction.

We further examined the role olfaction plays in male detection and location of females, and while we found no evidence that males are utilizing olfaction to find mates, we did observe behavioral changes to conspecifics of both sexes, and a reduction of activity in the presence of volatile cues from a predator. At levels of competitor cues that males are expected to encounter in nature, we found no evidence that male-male inhibition occurs in this species.

In examining male response to substrate bound cues, we found that males are able to discern female trails, and will preferentially follow them, although they are not able to determine the direction the trail was laid. We also utilized a custom built silk collection device to obtain sufficient quantities of clean silk for extraction and preliminary chemical analysis. Following solvent extraction of cues from the silk, we were able to reestablish searching behavior in response to the extract from female silk, but it did not elicit courtship display, suggesting a multicomponent chemical signal involved in sexual signaling in this species.

Dedication

To Chrissy Bell and the rest of my supportive family.

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I am deeply grateful to the support of many people during my educational journey, from the many teachers of my youth that inculcated a love of science and desire to understand the world around me, to my advisor, J.A. Roberts who has pushed and guided me towards being a better scientist and person. Among the rest of the Roberts lab, thanks go first to my lab mate S. Herrmann, as well as the many undergraduate students who have helped out in all aspects of maintaining large spider populations in the lab. My thanks also extend to the faculty and graduate students of the Department of Evolution, Ecology and Organismal Biology at The Ohio State University, for many discussions and opportunities to indulge my desire to learn about the natural world.

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Fields of Study

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Chapter 1: The Role of Olfaction in *Schizocosa ocreata*

ABSTRACT

Olfaction of airborne chemical cues may allow for detection of biologically important compounds such as pheromones at significant distances. Detection of airborne sex pheromones may aid in mate finding, and thus increase fitness through increased mating opportunities, while detection of volatile cues from a heterospecific predator may mitigate predation risk, increasing fitness through increased survivorship. We examined the response of male *Schizocosa ocreata* (Hentz 1844) wolf spiders (Lycosidae) to directional airflows containing conspecific and heterospecific cues, as well as response to conspecific and heterospecific cues in an enclosed arena with stationary air. While we found no evidence of males responding to olfactory cues in an active airstream, males did respond to cues in still air diffusing from stationary individuals. In response to conspecific cues, we found that males demonstrated an increase in display activity sufficient to suggest that there is olfactory detection. However, there were no detectable differences in response to male versus female conspecific cues, suggesting that the compound(s) detected are not sex specific. In still air containing volatile predator cues, males decreased overall locomotion and display activity relative to their response to conspecific cues, with response to blank controls generally intermediate to both.

INTRODUCTION

The sending and receiving of signals involved in intraspecific communication will rarely happen in the absence of other cues, as one’s environment may contain many stimuli generated by other organisms in multiple modalities, including visual, mechanical (acoustic and vibrational), and chemical stimuli. The transmission and reception of signals despite background noise provides the basis for communication, and gives these signals informational strength, causing an alteration of the behavior or physiology of the conspecific receivers (Bradbury & Vehrencamp 2011). The degree to which a species or individual will utilize these different modalities will be dependent on their morphology and the environment in which they live (Prokopy 1986; Hill 2001; Page & Jaeger 2004; Kulahei et al. 2008; Bradbury & Vehrencamp 2011). In a world awash in potentially informative stimuli, receivers will be attuned only to the small portion of cues that are ecologically relevant (Tomba et al. 2001; Greenfield 2002; Partan & Marler 2005).

Chemical signaling, of the available modalities, likely represents the earliest form of communication (Regnier & Law 1968; Wyatt 2003; Bradbury & Vehrencamp 2011), and is most taxonomically widespread (Greenfield 2002; Wyatt 2003; Thiel & Breithaupt 2011). Successful navigation of an individual’s chemical environment can greatly impact survival and eventual reproductive fitness (Tomba et al. 2001; Wyatt 2003; Bradbury & Vehrencamp 2011).

The compounds utilized in chemical signaling are broadly referred to as semiochemicals, and can be divided into pheromone signals when sender and receiver are of the same species, and allelochemical cues when they are of different species. A given semiochemical can also differ in how it is dispersed in the environment and encountered by the receiver. In terrestrial systems, compounds can be transmitted as an airborne chemical, or be deposited on a surface. Reception of an airborne component occurs through olfaction, potentially at a significant distance from the source, while contact chemoreception of a deposited compound requires direct contact between the receiver and a cue source (Wyatt 2003; Bradbury & Vehrencamp 2011).

The manner in which a particular chemical operates (contact or olfaction) is generally dictated by the chemical nature of the compound in question. Non-volatile, contact pheromones are typically high molecular weight, will not readily evaporate, and can only be detected by a receiver after contact with the source individual or surfaces upon which cues have been deposited (Wyatt 2003). Chemicals that operate in olfaction must be small (usually less than 300 molecular weight) and volatile, meaning they are highly dispersible in air, but also stable and at sufficiently high concentration to elicit a response from the receiver (Bossert & Wilson 1963; Regnier & Law 1968; Okubo & Levin 2001). Unlike visual or acoustic cues which can be quickly transmitted or propagated through the environment, airborne chemical cue molecules must physically travel by diffusion or along currents through the air from source to receiver. The net effect of this passive diffusion will be a gradual spread from areas of high to low concentration, while air currents (wind) will carry signal molecules directionally away from the sender, further increasing the rate and distance of dispersal. Airborne cues may be active over a long distance, and can allow for communication between spatially separated individuals. Like all other cues, airborne cues will have an active space, which in this case is the three dimensional space in which the cue is of sufficiently high concentration to elicit a response from the receiver (Kennedy & Marsh 1974; Okubo & Levin 2001; Greenfield 2002).

The dispersal pattern of volatile chemical cues tends to be slow, irregular, and highly dependent on the movement of air currents, and as such, temporal patterning is not a reliable means of encoding information (Wyatt 2003; Bradbury & Verhrencamp 2011). Instead, the chemical structure of the released compound(s) informs the receiver about the identity and status of the sender, while the concentration gradient may provide directional information (Wyatt 2003). Production and reception of more than one chemical cue increases the information that can be transmitted from sender to receiver (Greenfield 2002; Wyatt 2003). Additionally, a multicomponent signal comprised of a specific blend of chemicals may have a synergistic effect with deviations to the species-specific proportion of components rendering the signal behaviorally inactive (Symonds & Elgar 2008), as has been shown in arthropods (e.g. Lepidoptera, Linn et al. 1987; Phelan 1992; Coleoptera, Symonds & Elgar 2004) and mammals (Novotny et al. 1990).

Because the dispersal rate and direction of volatile compounds is reliant on diffusion and air movement, dispersal in natural environments with intermittent and shifting air currents may become quite complex (Bossert & Wilson 1963; Bradbury & Vehrencamp 2011). Many flying insect species have the ability to detect biologically important cues even in shifting and turbulent air, and fly to locate a potentially distant cue source (Farkas & Shorey 1972, Balkovsky & Shraiman 2002; Greenfield 2002). The detection of these airborne cues requires a concentration above an olfactory threshold (Greenfield 2002; Kennedy & Marsh 1974; Okubo & Levin 2001), and eventually the volatile cues will become too diffuse to elicit a behavioral response. Flying organisms can rapidly change position/direction in three-dimensional space to relocate transient odor plumes, but for non-flying, or slower moving organisms, responding to rapidly shifting volatile cues while constrained to terrestrial locomotion may be more difficult, and less likely to serve as a reliable means of finding the sender (Schulz 2004).

Olfactory chemical detection in spiders has been demonstrated to play a role in interspecific interactions, including avoidance of predators (Schonewolf et al. 2006) and finding prey (Allan et al. 1996). Some spiders produce volatile cues as a form of aggressive mimicry to attract prey (Stowe et al. 1987; Haynes et al. 1996), some use volatile cues to mediate intraspecific interactions such as aggregation (Furey & Riechert 1999), and many use them in sexual communication and mate finding (Tietjen 1979; Olive 1982; Tietjen & Rovner 1982; Watson 1986; Miyashita & Hayashi 1996; Papke et al. 2001; Kasumovic & Andrade 2004; Schulz 2004; Becker et al. 2005). A volatile pheromone chemical structure has only been identified for a few species of web building spiders (Papke et al. 2001; Xiao et al. 2009; Chinta et al. 2010), usually those in which males are attracted to female webs as she reaches maturity, but none have yet been identified for cursorial (non-web building) spiders. The capacity for male wolf spiders (Lycosidae) to detect and respond to airborne female cues has been demonstrated in

Pardosa milvina (Searcy et al. 1999), and suggested for others (Tietjen 1979), but remains largely unexplored.

The wolf spider *Schizocosa ocreata* is a litter dwelling species found in eastern deciduous forests of the United States, and has been widely used as a model organism in the study of multimodal communication and sexual selection (Hebets & Uetz 1999; Uetz 2000; Uetz & Roberts 2002; Hebets & Papaj 2005; Stratton 2005; Hebets et al. 2011). Upon encountering a substrate recently inhabited by a female, a male typically responds by engaging in species specific courtship displays that contain visual and seismic components (Bristowe & Locket 1926; Uetz & Denterlein 1979; Stratton & Uetz 1981; Stratton & Uetz 1983; Hebets & Uetz 1999). Female detection of these courtship signals plays a role in her receptivity and mate choice decisions (Uetz & Roberts 2002; Hebets & Papaj 2005). Due to the complexity of the leaf litter environment (Uetz 1979; Cady 1983), conspecific individuals may be spatially close, yet isolated in the visual and vibrational modalities (Scheffer et al. 1996; Elias et al. 2006; Gordon & Uetz 2011; Uetz et al. 2013). Communication through chemical cues may allow for the sending and receiving of information between these otherwise isolated individuals.

While contact chemoreception has been well established in *S. ocreata* (Uetz & Denterlein 1979; Roberts & Uetz 2004a, b; 2005), the possibility of olfaction has only been weakly suggested (Tietjen 1979). If olfactory communication is present in *S. ocreata*, it would greatly increase the information available to a courting male, providing a selective advantage in locating potential mates in a polygynous scramble competition mating system (Norton & Uetz 2005). The presence of volatile compounds should be a reliable indicator of the presence of a female, since with increasing distance the concentration of airborne components would likely become attenuated below some detection threshold. Male reception of female volatile cues likely is not sufficient to elicit full courtship displays (Tietjen 1979), but may play a role in mate-finding directly via chemotaxis, or indirectly by modifying male kinetic responses in a way that increases his encounter rate with females, substrate bound pheromones, or some other indication of the presence of a female spider.

While reception of female olfactory cues may elicit a male response, engaging in courtship behaviors without a receptive female nearby may be costly for males, both energetically (Cady et al. 2011) and through increased predation risk (Pruden & Uetz 2004; Roberts et al. 2006; Hoefler et al. 2008), which may further select for information gathering regarding proximity of females. Because sexual cannibalism is relatively common in spiders (Elgar 1992; Persons & Uetz 2005; Pruitt & Riechert 2009), selection is high for males to engage in distinctive courtship behaviors that identify them as conspecifics without greatly increasing risk of predation (Elgar 1992; Pruden & Uetz 2004). *Schizocosa ocreata*, like many other spiders, exhibits some sexual size dimorphism (Dondale & Redner 1990; Walker & Rypstra 2002, Wilder & Rypstra 2008), and pre-copulatory sexual cannibalism on males has been documented (Norton & Uetz 2005; Persons & Uetz 2005). The risk of being consumed by a potential mate is an additional selection pressure upon males (Persons & Uetz 2005), and being able to reliably detect the presence of nearby females has implications not just in terms of securing successful mating, but also for survival and future fitness. The fitness consequences for a male preyed upon after mating are clearly not as severe as one that succumbs to pre-copulatory cannibalism (Elgar 1992), so there should be strong selection pressure on males to detect the presence of a female spider from a distance, which may represent both a potential mate and a potential predator.

The presence of female volatile cues may indicate a hidden female is nearby, and potentially be used to locate her within the environment. In detecting and responding to female volatiles, males may exhibit a taxis response such as following a concentration gradient (as suggested by Tietjen 1979), or utilizing chemical signals in conjunction with air current directionality to move towards the female (Miyashita & Hayashi 1996). Alternatively, males may exhibit a kinesis response such as altering the speed or rate of turning, also increasing his residence in air currents containing female volatiles. However, detection of these cues may instead be of limited utility in locating her due to shifting wind currents moving through a structurally complex habitat. If males are unable to use female cues to locate her directly, reception may elicit generalized searching and chemoexploratory behavior, increasing his encounter rate with females or at least with substrate bound pheromones, which may be more informative in locating a hidden female.

Detection of heterospecific volatile cues emitted from a predator may mediate the antipredator response of an individual (Wyatt 2003; Hermann & Thaler 2014). For *S. ocreata*, early olfactory detection of the larger co-occuring intraguild predator, *Tigrosa helluo*, may be under strong selection. *Tigrosa helluo* habitat overlaps with that of *S. ocreata*, and they exhibit a predatory response to *S. ocreata* male display activity (Pruden

& Uetz 2004). When presented with substrate containing *T. helluo* cues, *S. ocreata* males significantly decrease display behaviors (Roberts & Uetz 2004b; Fowler-Finn & Hebets 2011, Nickley & Roberts unpubl.). While volatile cues of *T. helluo* have been shown to elicit an antipredator avoidance response in other species of wolf spider (Schonewolf et al. 2006), the response of *S. ocreata* to volatile cues of this predator has not previously been assessed.

Being able to detect volatile cues from potential mates as well as predators would provide male *S. ocreata* with information about other individuals in their local environment, and mediate their courtship and antipredator behaviors. In these studies we test the hypothesis that male *S. ocreata* are able to detect and respond to these volatile cues with behaviors that would benefit their fitness, either by locating a female directly, by eliciting behaviors that increase their probability of gathering additional information about her location, or by avoiding predation, thereby extending their time to find a mate.

METHODS

Spider collection and rearing

We collected the *S. ocreata* and *Tigrosa helluo* spiders used in these studies as immature individuals at The Dawes Arboretum, Newark, Ohio, USA (N 39.973863, W -82.40128). We collected the spiders used in Experiment 1 during April and October 2011, and the spiders used in Experiment 2 in April 2012 and 2013. All of the spiders we used were raised in the lab to maturity while housed in individual round plastic containers (*S. ocreata*, 500ml, 9cm diameter; *T. helluo*, 1000ml, 9cm diameter) on a moist coconut fiber substrate to provide water *ad libitum*. We fed spiders *Acheta domestica* or *Gryllodes sigillatus* cricket nymphs twice weekly (*S. ocreata* 2-3 nymphs; *T. helluo* 4-6 nymphs), and maintained them at room temperature (22-25°C) on a 13:11 h light:dark cycle.

Experiment 1: Male response to volatile cues in directional airflow

In order to establish whether focal males will spend more time in the presence of conspecific volatile cues, we exposed them to simultaneous air streams in an enclosed arena (15.4cm x 9cm x 6.3cm high, Fig. 1.1). We constructed the apparatus of plastic pipe (polyvinyl chloride, 3.4cm dia) so the single incoming air stream was split before passing through two stimulus chambers (containing stimulus individuals as appropriate), two laminar flow chambers packed with small diameter plastic tubes (15cm length x

0.3cm dia), entering the test arena, and exiting through the dual outflows, before being recombined to exit the system through the single outlet (Fig. 1.1). Passing the air through chambers packed with small diameter tubes immediately prior to the airstream entering the test arena created laminar airflow which helped us eliminate turbulence in the air stream within the arena.

Achieving laminar flow without cross mixing is also dependent on flow velocity (Reynolds 1883), so we used a variable-voltage DC power supply (Mastech HY3005F-3) to control two small computer fans (HP Compaq #UDQFWZH03-1N) which we placed at the single intake and single outflow of the system. We adjusted the voltage supplied to each fan (1.5V) to establish the minimal level that provided an even flow with no mixing of airstreams or turbulence within the arena, which we confirmed visually using CO_2 vapor (from dry ice) passed through the system prior to the start of experiments.

Flow rate was constant and below 0.6m/s, the detection threshold of our instrumentation (Kestrel 3000 windspeed meter). We covered the top of the test chamber with clear Plexiglas to prevent spider or airflow escape while still allowing video recording. We used a fresh sheet of white paper below the arena chamber for each trial to provide a contrasting substrate for the test arena that could easily be disposed of between trials. We cleaned the arena and test chambers with 99% ethanol and a Kimwipe to remove all traces of silk and chemical cues from the apparatus, then allowed the apparatus to dry completely before use in the next trial.

We presented focal males with either a control treatment of no cues in either airstream (N = 16), or no cues in one stream versus conspecific volatile cues from female

(N = 15), male (N = 16), juvenile (N = 15), subadult female (N = 15), or volatile cues from adult female *Tigrosa helluo* (N=15), a co-occurring wolf spider and known predator of *S. ocreata*. *Tigrosa helluo* has previously been demonstrated to produce an airborne chemical cue that results in avoidance behavior by smaller wolf spiders (Schonewolf et al. 2006), making it a good candidate for predator airborne cues.

Adult *S. ocreata* were used 2-6 weeks after their final adult molt. For the two immature conspecific treatments, we selected subadult females based on observation of early development of female sexual characters (distinct epigynum, lack of enlarged pedipalps), which are visible 1-2 molts prior to adulthood. We considered immature spiders to be juveniles if they were too young to determine sex (3+ molts prior to adulthood). The no cue treatment provided us with a control for potential side bias, and for each regular trial, we randomly selected a stimulus individual and assigned that individual randomly to one of the two stimulus chambers. A piece of mesh screen upstream from the chamber and the laminar flow tubes downstream kept the stimulus spider contained.

We introduced focal males at the downstream end of the arena, centered between the two airstreams, and allowed them to move about while being video recorded (Canon VIXIA HF S100 HD video camera) during the ten minute trial period. We scored trials from video playback using JWatcher V1.0 for duration of time spent in treated or untreated air streams. Because introducing males may have generated localized turbulence and prevented sufficient exposure to both airstreams, we only scored males that entered both airstreams during the trial. We scored the movement of focal males between treated and untreated air streams within the laminar flow arena, and used a one sample t-test to compare the mean total duration of time they spent in each airstream to a hypothetical mean of 300 seconds, corresponding to half the total trial duration.

Experiment 2: Male response to volatiles in stationary air

To assess male *S. ocreata* response to volatile cues spreading by simple diffusion (no directional airflow), we created a vertical, split-chamber apparatus where the columnar chamber was divided by a perforated screen allowing volatiles to move between chambers but isolating focal and stimulus individuals from interacting visually or vibrationally (Fig. 1.2). Specifically, in a one-way ANOVA design, we randomly selected stimulus (cue source) individuals from the lab population (*S. ocreata* used 1-4 weeks post maturity) for four treatment groups that included males (N=16), females (N=16), predators (*T. helluo*, N=15), or blank control (N=15). We placed stimulus individuals in small, mesh covered plastic tubes (polyvinyl chloride, 3.4cm dia, 5cm length) 24 hrs prior to use. At the start of a given trial, we placed a cue tube (containing stimulus individual or blank control) within an opaque plastic ring (polyvinyl chloride, 10cm dia, 5cm high) on a marble slab (for vibration isolation). We inverted a stainless steel soil sieve (Cole Parmer No. 60, 250µm mesh, 20cm dia) over the plastic ring and cue tube, then placed a clear, Plexiglas ring (10cm dia, 5cm high) on the mesh aligned with the plastic ring below. This ring provided a filming arena/test chamber for the focal males (Fig. 1.2). We covered the top ring with a Plexiglas sheet and allowed the apparatus to sit, undisturbed, for five minutes (allowing for cue diffusion before the start of a trial).

To start a trial, we randomly selected a focal male (1-4 weeks post maturity) from the lab population, moved the cover just enough to the side to lightly deposit the male onto the mesh screen (start of measured trial), and gently replaced the cover. Trials were video recorded (Canon VIXIA HF S100 HD video camera) from the side for five minutes, after which we returned the focal and stimulus individuals to their home containers. As in Experiment 1, we wiped down all components of the apparatus with 99% ethanol, then allowed them to dry completely prior to subsequent trials. We scored videos using JWatcher V1.0 for the frequency (total number of bouts per 5min trial) and total duration (total time engaged in behavior per 5min trial) of several behaviors previously demonstrated as critical in mediating conspecific and antipredator interactions. These include simple locomotion, active exploratory behavior (chemoexplore), display and courtship behaviors (tapping and jerky tapping, respectively), and antipredator behavior (stationary) as described by Delaney et al. (2007). We square root transformed frequency data and log transformed total duration data for analysis to meet the assumptions of ANOVA. We used a Bonferroni corrected alpha (α=0.01) in tests for significance, with post-hoc comparisons made using Tukey-Kramer HSD.

RESULTS

Experiment 1: Male response to volatile cues in directional airflow

We did not find a significant difference between time spent in the cue treated airstream versus the air stream containing no cues for any of the treatments (Female $t_{(14)} = 1.0$, $p = 0.323$; Male $t_{(15)} = 0.3$, $p = 0.805$; Juvenile $t_{(14)} = 0.5$, $p = 0.595$; Subadult $t_{(14)} = 0.7$, $p = 0.469$; No cue control $t_{(15)} = 0.6$, $p = 0.556$; *Tigrosa helluo* $t_{(14)} = 0.012$, $p = 0.99$). Since neither side of the control contained volatile cues, control trials were scored by side (left and right).

Experiment 2: Male response to volatiles in stationary air

Complete ANOVA results are presented in Table 1.1. We found a significant influence of volatile cue source on the frequency and duration of tapping behavior, frequency and duration of bouts of stationary behavior, and also on the frequency of bouts of locomotion (Table 1.1, Figs. 1.3 - 1.7). Post hoc comparisons across treatments revealed that male spiders displayed tapping behavior more often and for longer periods in response to conspecific cues, though there were no detectable differences between response to male versus female conspecific cues or between predator cues and blank controls (Figs 1.3 & 1.4). With respect to stationary behavior, males were stationary for fewer but significantly longer bouts in response to predator cues than in response to conspecific cues, with response to blank controls intermediate to conspecific and heterospecific cues (Figs. 1.5 & 1.6). The frequency of bouts of locomotion followed a similar pattern (Fig 7).

DISCUSSION

Olfaction of volatile chemicals has been established in many taxa, and can serve an important role in detection of sex pheromones. In the scramble competition for mates that male *S. ocreata* are faced with, the use of olfactory components in their communication system would potentially have strong fitness benefits if detection of female volatiles leads to increased mating success. However, our analysis shows that males are no more likely to remain in an airflow that has passed over a female than an airflow containing no spider cues, giving no indication that males are able to locate females using volatile cues in moving air. This result is not entirely unexpected given the complex leaf litter habitat of *S. ocreata* (Scheffer et al. 1996). If a female is hidden within the leaf litter, the spatial structure of the litter itself and forest floor air currents would result in turbulence and eddying as cues move through the vegetation and debris. Female volatile cues therefore, most likely provide little or no reliable, directional information and are thus of limited utility for locating the female directly.

Similar to the results for female cues, in the presence of other conspecific cues, focal males did not spend any more or less time in treated versus untreated air streams than would be predicted by chance. We did not expect juvenile or subadult treatments to elicit a male response, as they do not represent a potential mating opportunity, but cues produced by a male conspecific may represent a potential competitor to be avoided, though our study showed no indication of avoidance by focal males. Control trials did not reveal any effect of apparatus side bias.

We also explored male response to potential volatile cues of a co-occuring intraguild predator, *Tigrosa helluo*. Male *S. ocreata* have been shown to decrease display behaviors in the presence of silk from this larger wolf spider (Roberts & Uetz 2004b; Fowler-Finn & Hebets 2011, Nickley & Roberts unpubl.), but we found no evidence that males in our study were detecting or responding to directional volatile emissions from stimulus *T. helluo*.

In the laminar flow studies, even though air flow was lower than the detection threshold of our equipment, it is possible that the constraints of our apparatus did not allow us to set the air flow low enough for volatile compounds to disperse within the arena above a biologically relevant threshold. If signaling chemicals were being too highly attenuated in the air stream, they may have been below the level at which our focal males could detect them.

Experiment 2, with males and source individuals enclosed in essentially a single chamber, provided a measure of control over this limitation. Focal males were enclosed in an arena with only a layer of fine stainless steel mesh between them and the source of volatile cues, but no directional flow (passive diffusion only). We did find a significant effect of treatment on mean frequency of bouts and mean total duration of tapping and remaining stationary by the focal males, as well as an effect of treatment on mean frequency of locomotion bouts (Table 1.1), though active courtship (jerky tapping) was conspicuously absent. We found that male tapping responses to both male and female volatile treatments were significantly different from responses to predator cues in all post hoc pairwise comparisons (Fig. 1.3 - 1.7). Tapping was elevated in duration and frequency in treatments where focal males were exposed to conspecific volatiles (either male or female) compared to the control or *T. helluo* (Figs. 1.3 & 1.4). This potentially suggests a chemical signal indicating the presence of a conspecific, but not the sex of the sender.

The presence of *T. helluo* volatiles decreased the frequency of focal spider movement behaviors (stationary and locomotion), and increased the amount of time spent stationary compared to those exposed to conspecific volatiles (Figs. 1.5 - 1.7). However, behavioral responses of males in *T. helluo* treatments did not differ from males in the control treatment, suggesting that volatile cues indicating the presence of the larger predator did not have a strong effect. The low level of activity in the presence of *T. helluo* cues may be an indication of a detectable volatile cue being produced by this predator, and while the effect may not be strong, any reduction in predator attack would prove a selective advantage for the male.

Our behavioral assays of male *S. ocreata* olfactory response to volatile cues does not provide support for the presence of any female specific, volatile sex pheromones. We did find evidence of increased tapping activity by males, which may be a response to a volatile component produced by both sexes. There is the possibility that female specific volatile pheromones do exist in this species, but that their reception does not elicit immediately observable changes in the receiver. Reception of a volatile pheromone could serve to prime the male, either physiologically or for future behavioral responses.

Additionally, we did find some evidence that male *S. ocreata* are able to detect the presence of *T. helluo* by airborne allelochemicals. While this did not result in a negative taxis response in the laminar flow chamber, we did observe a significant reduction in activity in the enclosed chamber when presented with *T. helluo* volatile cues compared to conspecific cues (Table 1.1). However, activity in the presence of *T. helluo* cues was never significantly different from the blank control (with no spider cues), suggesting that any airborne *T. helluo* cues alone were not strong enough to elicit a complete antipredator response as described in previous studies (Roberts & Uetz 2004b; Fowler-Finn & Hebets 2011).

The increased activity in the presence of conspecific cues may be indicative of an increase in kinesis behavior, which may result in increased encounter rates with conspecific individuals or other chemical cues. A potentially informative future direction for investigation of olfaction in this species would be the use of video tracking software to explore whether detection of volatile cues impacts the rate of locomotion and distance traveled, as this would represent another aspect of increased kinesis not able to be scored from this study.

If olfaction is of limited functionality within the complex leaf litter habitat in which *S. ocreata* is native, it is possible that evolution of a sophisticated olfactory system able to locate potential mates simply was not selected for in this species. While olfactory communication being used to locate mates in this species had previously been suggested (Tietjen 1979), our studies did not find further direct evidence in support of this finding, but did show that males increase activity in the presence of conspecific cues and decrease activity in the presence of predator cues.

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	Total Duration			Total Number		
	F	df	p	F	df	p
Locomotion	3.2	3,58	0.03	7.8	3,58	0.0002*
Stationary	4.8	3,58	0.0046*	8.3	3,58	0.0001*
Chemoexplore	0.8	3,58	0.50	0.6	3,58	0.59
Tapping	9.3	3,58	<0.0001*	10.3	3,58	<0.0001*
Jerky Tapping	0.9	3,58	0.44	1.1	3,58	0.36

* indicates significance following Bonferroni correction ($\alpha = 0.01$)

Table 1.1. ANOVA results for duration and frequency of male behaviors in the enclosed arena with stationary air.

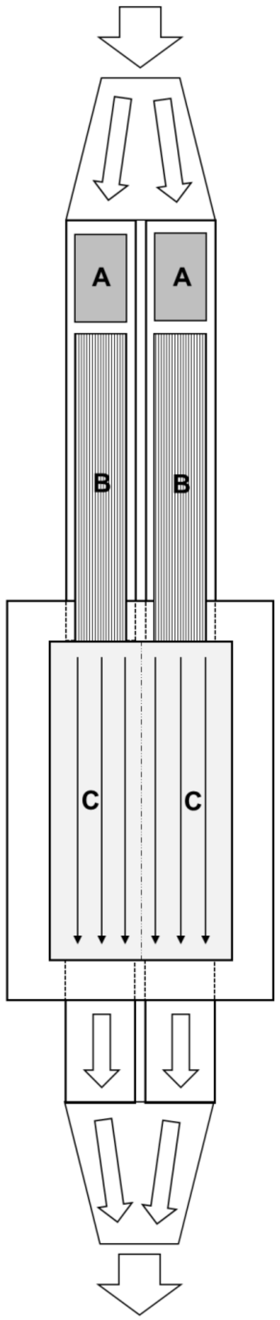

Figure 1.1 Directional airflow apparatus with laminar flow. Arrows indicate the direction of airflow, thick arrows indicate bulk airflow and thin arrows represent laminar flow within the test arena. A) Cue stimulus chamber, B) laminar airflow baffle, C) test arena with separate air streams (distinction between air streams indicated by dashed line in C).

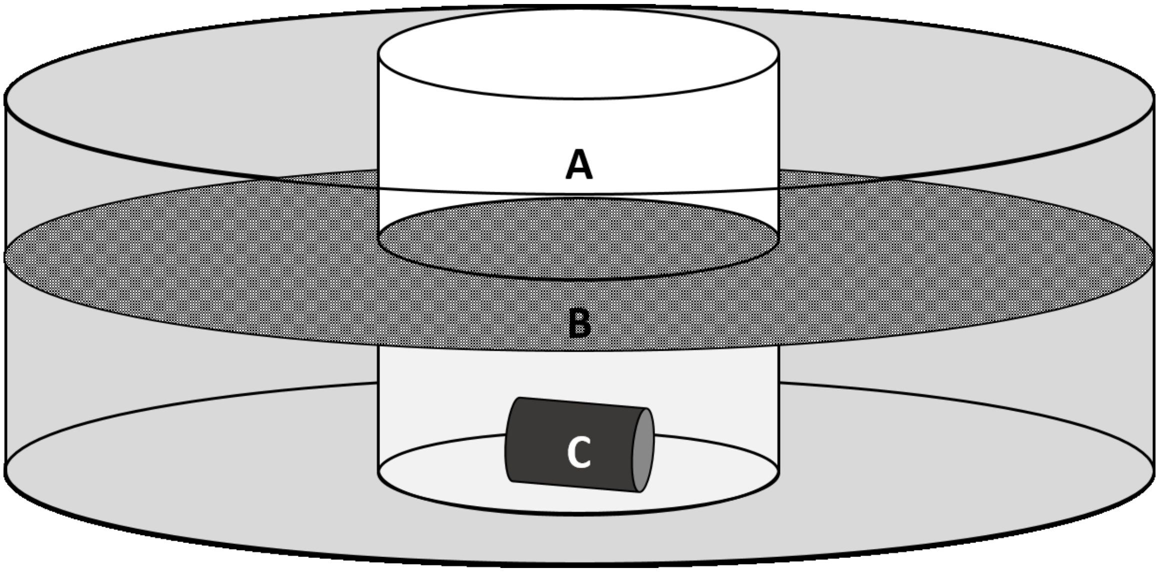

Figure 1.2. Enclosed arena design for assessing male *Schizocosa ocreata* response to volatiles in stationary air. A) clear Plexiglas upper chamber where focal males were observed, B) wire mesh substrate, C) lower chamber containing isolated stimulus individual.

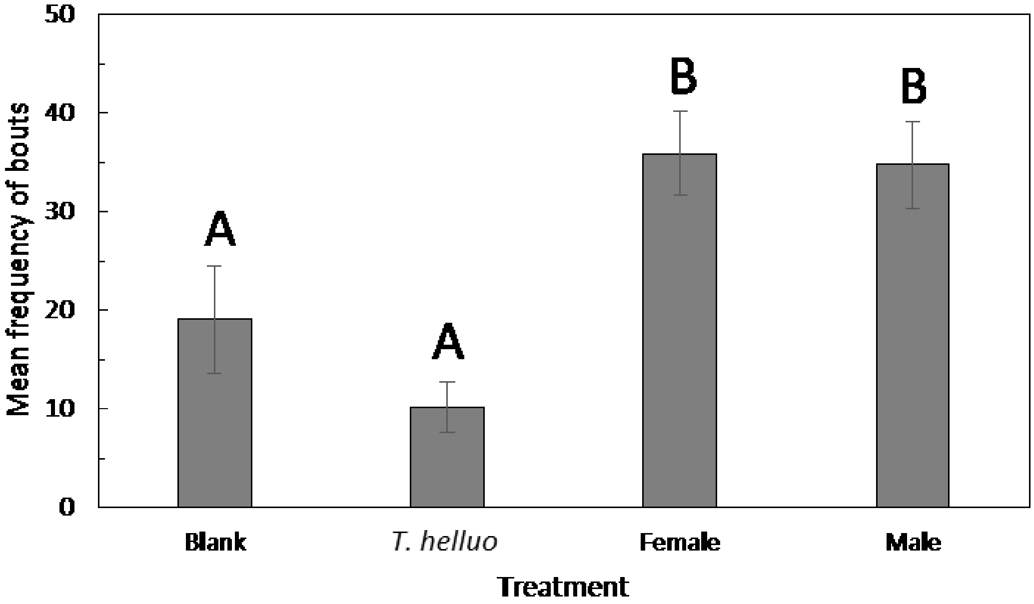

Figure 1.3. Mean frequency of bouts (± SE) of tapping behavior for male *Schizocosa ocreata* exposed to volatile cues of visually and vibrationally isolated stimulus individuals. Shared letters above the bars indicate no significant difference by Tukey-Kramer post hoc analysis.

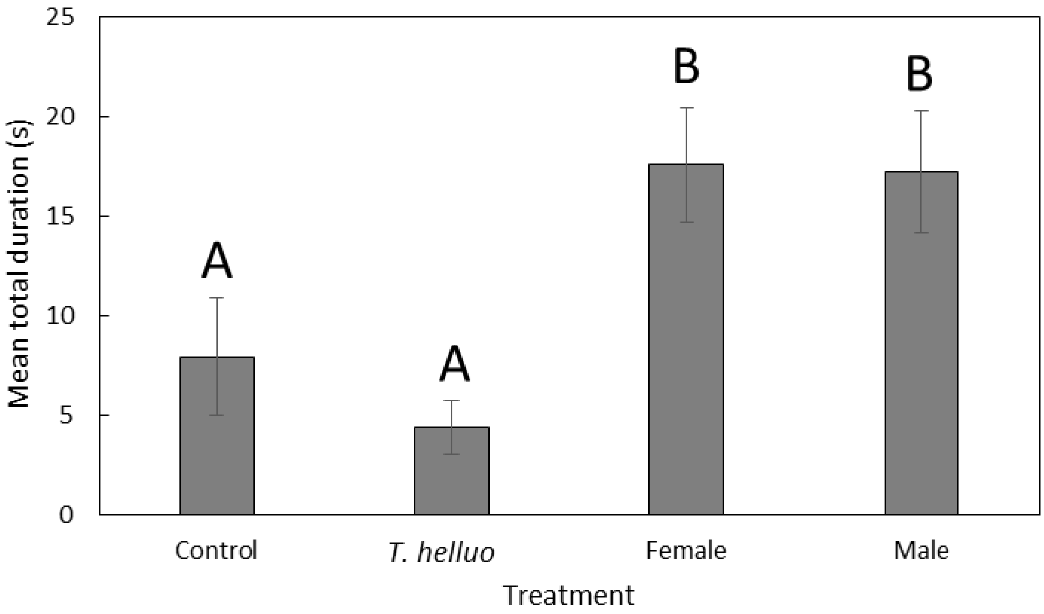

Figure 1.4. Mean total duration (s) (± SE) of tapping behavior for male *Schizocosa ocreata* exposed to volatile cues of visually and vibrationally isolated stimulus individuals. Shared letters above the bars indicate no significant difference by Tukey-Kramer post hoc analysis.

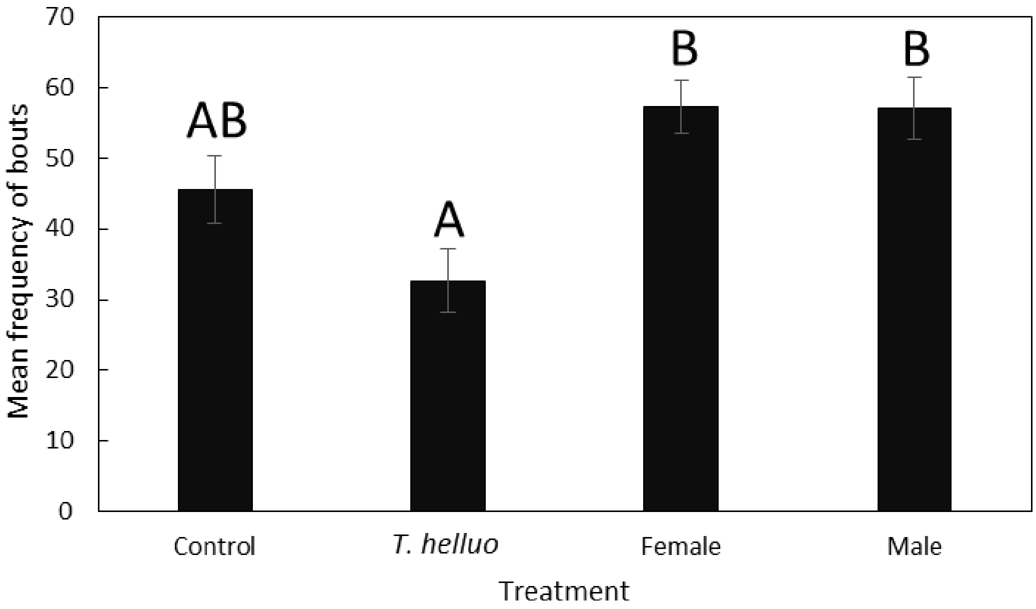

Figure 1.5. Mean frequency of bouts (± SE) of stationary behavior for male *Schizocosa ocreata* exposed to volatile cues of visually and vibrationally isolated stimulus individuals. Shared letters above the bars indicate no significant difference by Tukey-Kramer post hoc analysis.

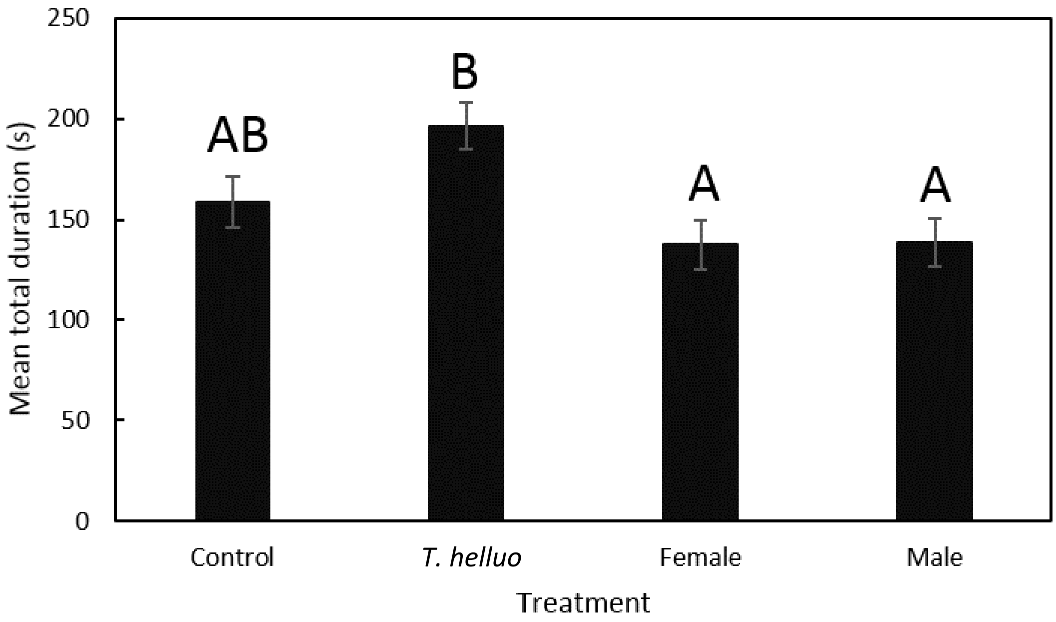

Figure 1.6. Mean total duration (s) (± SE) of stationary behavior for male *Schizocosa ocreata* exposed to volatile cues of visually and vibrationally isolated stimulus individuals. Shared letters above the bars indicate no significant difference by Tukey-Kramer post hoc analysis.

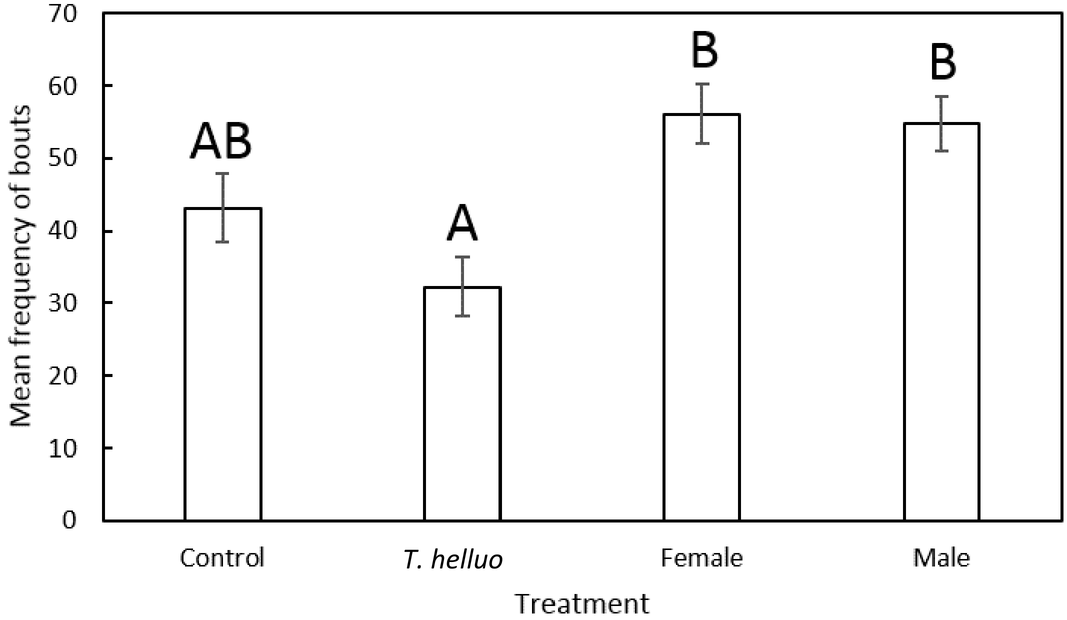

Figure 1.7. Mean frequency of bouts (± SE) of locomotion behavior for male *Schizocosa ocreata* exposed to volatile cues of visually and vibrationally isolated stimulus individuals. Shared letters above the bars indicate no significant difference by Tukey-Kramer post hoc analysis.

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Chapter 2: The Potential Role of Male-Male Inhibition in *Schizocosa ocreata*

ABSTRACT

Male competition can extend beyond the physical to include chemical competition via inhibition of male behavior or reduction of another male's ability to detect relevant cues. The existence of a male-male inhibitory pheromone has previously been proposed in the wolf spider *Schizocosa ocreata* (Hentz 1844), but was demonstrated only under conditions exceeding naturally occurring cue concentrations, leaving a question of whether this inhibitory effect has any ecological relevance. We examined the response of focal males to concentrations of competitor cues that are more comparable to what they would experience under field conditions, both in the presence and in the absence of female cues. Our studies found no evidence to support the presence male-male inhibitory pheromones in this species.

INTRODUCTION

Chemical cues, such as pheromones, serve an important role in sexual communication of many species (Wyatt 2003), allowing for identification and location of potential mates. In a natural system, the context in which these cues are received can be quite chemically complex where the cues of a potential mate can co-occur with other detectable chemicals produced by heterospecifics (prey or predators) and conspecific competitors. Prioritization of behavioral response to cues from a potential mate versus other sources may represent a tradeoff between finding a mate and securing prey or avoiding predation. Response to chemical cues from, or signal interference by another male could represent a form of competition, and influence interactions with that conspecific competitor, as well as potential mates sought by both suitors. Though best known for eliciting or amplifying behaviors in a receiver, it is possible for chemicals produced by conspecifics to have inhibitory effects on the receiver. Inhibition, in the context of sexual chemical communication, refers to actions or products of one individual that reduce or prevent the response of a second individual to otherwise behaviorally active, informative stimuli. Use of inhibitory pheromones has been found in maintaining social hierarchies, with long term physiological impacts on the receivers in mammalian systems (Perret 1992; Stoddart et al. 1994), as well as eusocial insects (Strauss et al. 2008).

In systems with a chemically signaling female, males may engage various forms of inhibition to prevent other potential suitors from detecting or responding to the female cues. This may be via the production of male specific compounds that reduce or attenuate female pheromone attractiveness when received concurrently, as has been shown in the moth *Adoxophyes orana* (Bijpost et al. 1985; Den Otter et al. 1989). Alternately, Bijpost et al. (1985) suggested the possibility for systems involving a multicomponent pheromone signal to be disrupted by the production of one or more components of the signal, skewing the ratio outside the behaviorally active blend. A similar concept may be deployed in mating disruption of commercial pest species, inundation of the area with a component of the pheromone blend eliminates the ability of males to find signaling females (Gaston et al. 1967; Cardé & Minks 1995). Inhibition of a chemical signal can even be achieved through behavioral pathways, as found in the spider *Linyphia triangularis*. In this species, pheromones are present in the silk of the female web, and the first male to find a receptive female begins deconstructing her web, reducing its surface area and signal strength to other males (Watson 1986; Schulz & Toft 1993).

Inhibition can also occur directly between males. Male *D. melanogaster* produce cuticular hydrocarbons found to repel other males, the suggested function being deterrent of unintended mounting by competitors (Lacaille et al. 2007). These compounds are also transferred via contact with females during mating (Scott 1986), along with accessory compounds present in the sperm that reduce her receptivity and induce female production of the same cuticular hydrocarbons, reducing her attractiveness to future suitors (Scott

1986; Kalb et al. 1993). The role of inhibitory compounds in altering the behavior of the receiver may have evolutionary benefits to both the sender and the receiver by reducing direct competition and energy expenditures involved in continued searching for a mate who may no longer be receptive.

In the mating system of *Schizocosa ocreata*, a small wolf spider (Lycosidae) common in leaf litter of eastern deciduous forests of North America (Dondale & Redner 1990), polygynous males engage in a scramble competition for access to receptive monandrous females (Norton & Uetz 2005). Detection of female chemical cues associated with dragline deposition results in male courtship displays in lycosid spiders (Hegdekar & Dondale 1969; Tietjen & Rovner 1982; Pollard et al. 1987). Females convey information about species identity and mating status to males via chemical cues (Roberts & Uetz 2004a,b; 2005), while male visual and seismic courtship serves both to advertise his identity as a male of the species, and influence her propensity to mate with him (Uetz 2000; Uetz & Roberts 2002; Hebets & Vink 2007). In addition to interacting with potential mates, male *S. ocreata* may interfere with one another, engaging in male-male contests (Aspey 1977a; Delaney et al. 2007), or engaging in courtship display and signal matching when they observe other courting males (Clark et al. 2012).

Ayyagari and Tietjen (1986) demonstrated dramatic inhibitory effects on the behavior of male *S. ocreata* using chemical extracts taken from the silk of conspecific males. *Schizocosa ocreata* do not build webs and instead, both sexes deposit dragline silk as they move through the environment. The potential for simultaneous deposition of an inhibitory chemical in male silk has interesting implications for social interactions, as it may be present wherever a male spider has been. In the study by Ayyagari and Tietjen (1986), focal males froze entirely in the treatment with the highest concentration of silk extract, and post exposure refused to respond with courtship behaviors even when exposed to female cues alone. In developing the extract from male silk, they used treatments ranging from 60 - 696 silk days (pooled extracts from multiple spiders present on substrates for multiple days).

In *Schizocosa*, chemical cues deposited with female silk are resistant to degradation by some abiotic variables, but highly susceptible to quick and lasting inactivity by moisture, suggesting a polar compound (Roberts & Uetz 2004a; Baruffaldi et al. 2010; Roberts unpubl.). Rainfall or dew alone would therefore likely serve to reset the pheromone landscape with regards to female pheromones. Due to the degradation of signaling compounds in silk under natural conditions, it is likely that the concentrations used by Ayyagari and Tietjen (1986) are far in excess of what is possible in nature, and thus may not be biologically relevant. In their work on ant alarm pheromone systems, Hölldobler & Wilson (1990) note that nearly any chemical component produced by ants may appear to serve an alarm function at sufficient concentrations, yet only those that have an effect at naturally occurring levels can be considered alarm pheromones. Similarly, an inhibitory effect may be observable at high concentrations, yet not correspond to an inhibition of male behavior at natural levels.

Investigation of any relevant role for a male inhibitory pheromone should be done at a more biologically appropriate cue concentration, essentially the amount that would be deposited in a single pass by any given male. If a male inhibitory compound is being produced and detectable at low thresholds, males may be able to distinguish between a female trail that has been walked over by competitor versus a trail that contains only female cues. Since males are able to follow detect and follow the trails of monandrous females (Bell & Roberts unpubl), each subsequent male may be at a fitness disadvantage if he expends energy attempting to locate an already mated female.

Residence of competitor males in an area at more density appropriate levels may further increase the amount of male inhibitory pheromone that accumulates. A given male is likely to encounter up to 20 other males per day (Roberts et al. unpubl). Detection of cues from multiple males, or the overall quantity of cues may serve as an indication of local density, and thus potential competition that may be encountered. In the current study we test the hypothesis that males produce a male-inhibitory pheromone that mediates male-male interactions. To that end, we seek to determine whether males show a propensity to avoid chemical cues from other males or alter their display behaviors under increasing levels of competitor cues comparable to what may be encountered in nature.

METHODS

Spider collection and rearing

We collected all *S. ocreata* spiders used in this study as immature individuals from The Dawes Arboretum, Newark, Ohio, USA (N 39.973863, W -82.40128) during October 2013 or April and October 2014 and raised them in the lab until used in trials. We housed all spiders in individual plastic containers (500ml, 9cm diameter, round). A moist coconut fiber substrate provided water *ad libitum*, and we fed each spider 2-3 cricket nymphs (*Gryllodes sigillatus*) twice weekly. Lab conditions were set a room temperature (22-25°C) and a 13:11 h light:dark photoperiod.

Experiment 1: Single male competitor trail discrimination

We established cue trails in a modified Y-maze (Fig. 2.1) by blocking one branch of the maze, and gently guiding stimulus spiders to the termination of the open branch, where we then removed them. Initial cue trails were deposited by a randomly selected female 1-5 weeks post maturity. We then blocked the opposite branch, reintroduced the same female, and guided her to the termination of the second branch and removed her. Following establishment of female cues, we randomly selected a cue male (2-8 weeks post maturity), and blocked off one arm before the male was introduced, guided down the open branch then removed. We allowed males to move forward through the Y-maze and engage in display behaviors, but in order to establish a single pass trail, held a section of tubing behind them to prevent them from moving backwards. For each trial, we randomized side order for first versus second run of female cues, as well as the side treated by male cues to control for any sequence or side bias.

We introduced focal males (2-5 weeks post maturity) at the release point of the modified Y-maze (Fig. 2.1), covered the maze with Plexiglass, and allowed them to move through the maze during a ten minute trial while being video recorded (Canon VIXIA HF S100 HD video camera). We excluded any trials where the male never left the initial leg, and thus did not make a choice between the two arms of the maze. We scored as following or not following all trials in which males made a first turn choice (N=37), and those trials where the male completed the maze by entering an end chamber (N=31). We scored male first turn and first end chamber choices by Chi-square analysis under an assumption of no difference between sides of the maze. We also scored the mean duration of time spent in each treated arm during trials.

Experiment 2: Multiple male competitor trail discrimination

We established cue trails from females 1-4 weeks post maturity in both arms of the Y-maze, as described in Experiment 1, and randomly assigned each trial to zero male (N = 12), one male (N = 12), two males (N = 12), or three males (N = 12) treatments. For treatments containing male cues (2-5 weeks post maturity), an arm was randomly blocked off and we permitted randomly selected males to move through the Y-maze, removing them from the end chamber of the open arm. For treatments with additional male cues, we introduced stimulus males to the same open arm as appropriate for treatment, and permitted them to move through the maze.

We introduced focal males and permitted them to move through the maze while being video recorded (JVC Everio Camcorder GZ-HM30BU) during the ten minute trial. We later scored males on whether their first turn was into the arm with female cues only, or cues from one or more males. The zero male treatment provided a measure of control against the potential for side bias. Chi-square analysis was used to test whether treatment had an effect on the movement of males through the maze.

Experiment 3: Response to female cues in the presence of cues from multiple competitors

In the field, males have been observed to concentrate their courtship efforts on dwindling patches of leaf litter as the season progresses (Roberts & Uetz unpubl.). In order to simulate this in the lab and concentrate any male inhibitory compounds present to generate a stronger indication of potential competition, we used cue deposition over a longer time frame by multiple males. To create treatments of varying cue intensity, we established three levels of male cues (one male, two males, three males), along with a control containing no male cues, with all treatments containing female cues. We randomly selected both female and male spiders to be used in cue deposition 48 hrs post feeding to standardize hunger. To collect female cues, we placed a disc of filter paper (9cm) in the bottom of a clean glass Petri dish (9cm), and added a female (2-5 weeks post maturity) to deposit cues overnight. We removed the female the following morning, and placed the filter in a sealed plastic bag until trials were run. Filter paper containing female *S. ocreata* cues has been show to elicit normal male courtship response when stored similarly (Campbell & Roberts 2015).

On the day that trials were run, we established male cues in 1 hour increments, and for treatments containing cues from more than one male, a different male spider was used during each hour long increment. We placed a randomly selected disc of filter paper containing female cues in the bottom of a clear plastic container (9cm diameter, round), cue side up. To keep male spiders visually isolated from one another and prevent observation of other courting males, we placed each container within an opaque plastic ring (polyvinyl chloride, 10cm dia, 7.5cm high) before adding the males. During the first hour of male cue deposition, we added no spiders to the control treatment (N = 15), but otherwise handled the female treated filter paper the same as those to receive male cues. For all male cue treatments, we added a randomly selected male (4-10 weeks post maturity) to each container and allowed them to move around for an hour before we removed them. During the second hour, we did not add spiders to the one male treatment (N = 14), but we placed new, randomly selected male spiders on the filter paper of the two male (N = 13) and three male treatments (N = 15), and each was permitted an hour to deposit any cues. At the end of the hour, we removed all spiders, then we added new males to the three male treatments only, which we removed after another hour.

At the start of each trial, we placed a disc of filter paper within a clear Plexiglas ring (10cm inner dia) on a vibrationally isolated marble slab to prevent ambient seismic background noise from interfering with focal male response. We randomized the order of treatments to avoid any effects of time of day. We introduced a randomly selected focal male to the arena and covered the arena with a sheet of Plexiglas. Video recording each trial (5 min) from the side allowed us to later observe male behaviors, and score them using JWatcher (V 1.0). We log transformed duration of male behaviors for analysis, and used a Bonferroni-corrected critical value ($\alpha = 0.01$) to account for multiple comparisons in significance tests of the ANOVA results (Shaffer 1995).

Experiment 4: Response to cues from multiple competitors

The context in which any male inhibitory compounds are received may be important for how receiver males integrate that signal into their behavior. A series of trials were run where response males were presented with varying levels of male cues, but in the absence of any stimuli from female *S. ocreata*. All males were used at 1-8 weeks after their adult molt. We established male cues on fresh filter paper as in Experiment 3 to obtain a control treatment with no cues (N = 12), cues from one male depositing for one hour (N = 14), cues from two males depositing for one hour each (N = 13), and cues from three males depositing for one hour each (N = 10). Response males were presented with the treated filter papers in the same arena as Experiment 3 and were video recorded for a 4 min trial. We again scored behaviors with JWatcher (V 1.0), and tested log transformed duration with ANOVA at a Bonferroni-corrected critical value of $\alpha = 0.01$.

RESULTS

Experiment 1: When offered a choice between female cues alone versus female cues over which male cues had been laid in a modified Y-maze, focal *S. ocreata* males did not significantly differ in the first turn made ($X^2_{1,37} = 0.111$, $p = 0.739$) or the first end chamber entered ($X^2_{1,31} = 0.806$, $p = 0.369$). After initial choices were made, focal males did not spend a significantly different amount of time in either treated arm (Fig. 2.2).

Experiment 2: Although more focal males made their first turn into the arm containing male cues in all treatments, we did not find a significant difference between entering female only and male treated arms of the modified Y-maze. Males presented with female cues in both arms did not show a significant side bias ($X^2_{1,9} = 2.778$, $p = 0.096$). Nor did we find a statistically significant difference between female only and female plus male cues in the one male ($X^2_{1,10} = 0.40$, $p = 0.527$), two male ($X^2_{1,10} = 1.60$, $p = 0.206$), or three male treatments ($X^2_{1,10} = 3.6$, $p = 0.0578$).

Experiment 3: Focal males presented with filter paper containing female cues alone, or female cues masked by one, two, or three potential male competitors, demonstrated no difference in time spent engaged in movement behaviors (locomotion and stationary) or display behaviors involved in mate finding and courtship (chemoexplore, tapping, jerky tapping) (Table 2.1).

Experiment 4: When presented with filter paper containing no female cues, but cues from 0-3 male competitors, we found no difference in the duration of focal male movement and display behaviors (Table 2.2).

DISCUSSION

The ability to detect the presence and density of competitors has the potential to inform courtship decisions and expenditure of energy. If mating with virgin females confers a fitness benefit and detection of inhibitory compounds is an indicator of another male's mating success (Gilbert 1976; Scott 1986; Schulz et al. 2008; Brent & Byers 2011), then a reduced response to female cues in the presence of these compounds may be adaptive by reducing competition for a single female (Hirai 1978). If females show reduced receptivity to remating, or the second male to mate secures an extremely small paternal share of the offspring, his efforts may be better spent locating a receptive virgin mate.

Bijpost et al. (1985) proposed that male inhibitory pheromones could have an effect either directly by binding to or blocking chemoreceptors in the receiver, or indirectly by masking the female signal. Direct effects may result from the stimulation of a receptor specific to a male produced pheromone (Den Otter et al. 1989; Schulz et al. 2008; Brent & Byers 2011), or by interference with the reception and information processing of female cues. The addition of different isomer versions of female pheromones can have an inhibitory effect (Den Otter 1977; Löfqvist 1986; Anderbrant et al. 1992), potentially by interfering with the active isomer binding to receptors, or through binding to a specific receptor. In chemical signaling systems utilizing a multicomponent blend, delayed degradation within the receiver's sensory structures can effectively shift the proportion of the blend, potentially causing it to lose signaling function (Vogt 2003).

High concentration extracts of male *S. ocreata* silk have been shown to arrest behaviors during exposure, with effects that persist after removal of the stimuli, leading Ayyagai and Tietjen (1986) to propose the existence of a male inhibitory pheromone. However, their use of a concentration far beyond what would naturally occur makes it difficult to determine that the compounds males were responding to have any biological relevance or inhibitory function at natural levels. It is possible for any number of other metabolites or chemicals deposited by males to influence spider behavior given sufficient concentration (Hölldobler & Wilson 1990). In examining male response to cues established at more natural levels, we sought to establish whether males are detecting and responding to compounds deposited by other males that could be characterized as inhibitory pheromones.

In the studies presented here, we found no evidence for a biologically relevant male inhibitory pheromone in *S. ocreata* at competitor densities comparable to those found in nature. Any male cues deposited on the substrate did not result in preferential trail following when given the choice between female cues and female and male cues. Furthermore, in the presence of combined male and female cues, focal males show no reduction in activity across treatments relative to the control or female cues alone (Table 2.1). While cues of a potential mate and a potential competitor are present, it is possible that the cost of not engaging in a courtship display when encountering female cues is too great to result in a reduction in display behaviors. In *S. ocreata*, as in many spider species, courtship display as a means of advertising sex and species identity has a dual impact on the courting male's fitness when approaching a potentially cannibalistic female (Persons & Uetz 2005). While female *S. ocreata* are primarily monandrous (Norton & Uetz 2005), male courtship and chemoexploratory behaviors still persist in response to cues from mated females, though at a reduced intensity (Roberts & Uetz 2005). Within the complex leaf litter environment of *S. ocreata* males may frequently encounter silk from females of varying reproductive and receptivity states, but engaging in courtship behaviors may serve to mitigate female predatory response.

If a male compound is being produced and received, but does not constitute a sufficient inhibitory signal to reduce courtship in response to female cues, it might be expected to influence male behavior where female cues are not present. However, when presented with filter paper containing no conspecific cues, or cues from one to three males, there was no significant impact of treatment on response male behavior (Table 2).

It should be noted that not all pheromones detected by a receiver need be releaser pheromones resulting in a corresponding behavioral response, but may instead be primer pheromones, having a more subtle and long lasting effect on the physiology of the receiver (Wyatt 2003). Detection of the presence of competitors therefore may not have an immediate effect on movement and display behaviors of male *S. ocreata*, but may influence future social interactions.

At male cue densities that spiders would reasonably encounter in their natural environment, we found no evidence that the presence or absence of male cues influences male behavior in following female trails, in their response to female cues, or in their response to male cues alone. These studies suggest that the drastic example of male-male chemical inhibition observed by Ayyagari and Tietjen (1986) is likely an artifact of using a concentration greater than is biologically relevant. While we are not able to definitively rule out the existence of a male pheromone with inhibitory properties, if it does exist, it would seem to have little or no impact on male courtship behaviors at naturally occurring concentrations.

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Treatment	Locomotion		Stationary		Tapping		Jerky Tapping		Chemoexploration	
	Mean (s)	± SE	Mean (s)	± SE	Mean (s)	± SE	Mean (s)	± SE	Mean (s)	± SE
1M	76.76	11.94	106.52	10.95	6.76	3.96	27.88	9.49	53.87	8.70
2M	115.57	11.51	80.04	10.55	12.93	3.82	32.15	9.14	42.53	8.38
3M	61.38	11.94	88.32	10.95	7.42	3.96	29.25	9.49	49.22	8.70
FC	81.30	11.94	74.44	10.95	16.02	3.96	42.79	9.49	55.59	8.70
ANOVA	$F_{3,52}$= 3.84, p = 0.015		$F_{3,52}$= 1.6, p = 0.19		$F_{3,52}$= 1.27, p = 0.295		$F_{3,52}$= 0.51, p = 0.679		$F_{3,52}$= 0.47, p = 0.70	

Table 2.1. Mean duration (s) of movement and display behaviors when in the presence of filter paper containing female cues (FC) alone, or female cues and 1, 2, or 3 hours of deposited male cues (1M, 2M, 3M). Following Bonferroni adjustment of the alpha level (α = 0.01), there was not a statistically significant difference between any of the treatments.

	Locomotion		**Stationary**		**Tapping**		**Jerky Tapping**		**Chemoexploration**	
	Mean	± SE	Mean	± SE	Mean	± SE	Mean	± SE	Mean	± SE
1M	102.88	16.18	165.07	17.27	3.55	3.13	0.62	0.56	3.55	3.14
2M	60.39	16.79	194.21	17.93	6.07	3.25	0.45	0.58	6.07	3.25
3M	97.62	19.14	175.60	20.44	6.55	3.70	2.07	0.66	6.55	3.70
NC	56.40	17.47	218.38	18.66	4.56	3.38	0.00	0.61	4.56	3.38
ANOVA	$F_{3,48}$= 2.01, p = 0.13		$F_{3,48}$= 1.63, p = 0.20		$F_{3,48}$= 0.17, p = 0.92		$F_{3,48}$= 1.94, p = 0.14		$F_{3,48}$= 0.17, p = 0.92	

Table 2.2. Mean duration (s) of movement and display behaviors when in the presence of filter paper containing no cues (NC), or cues from 1, 2, or 3 hours of deposited male cues (1M, 2M, 3M). Following Bonferroni adjustment of the alpha level (α = 0.01), there was not a statistically significant difference between any of the treatments.

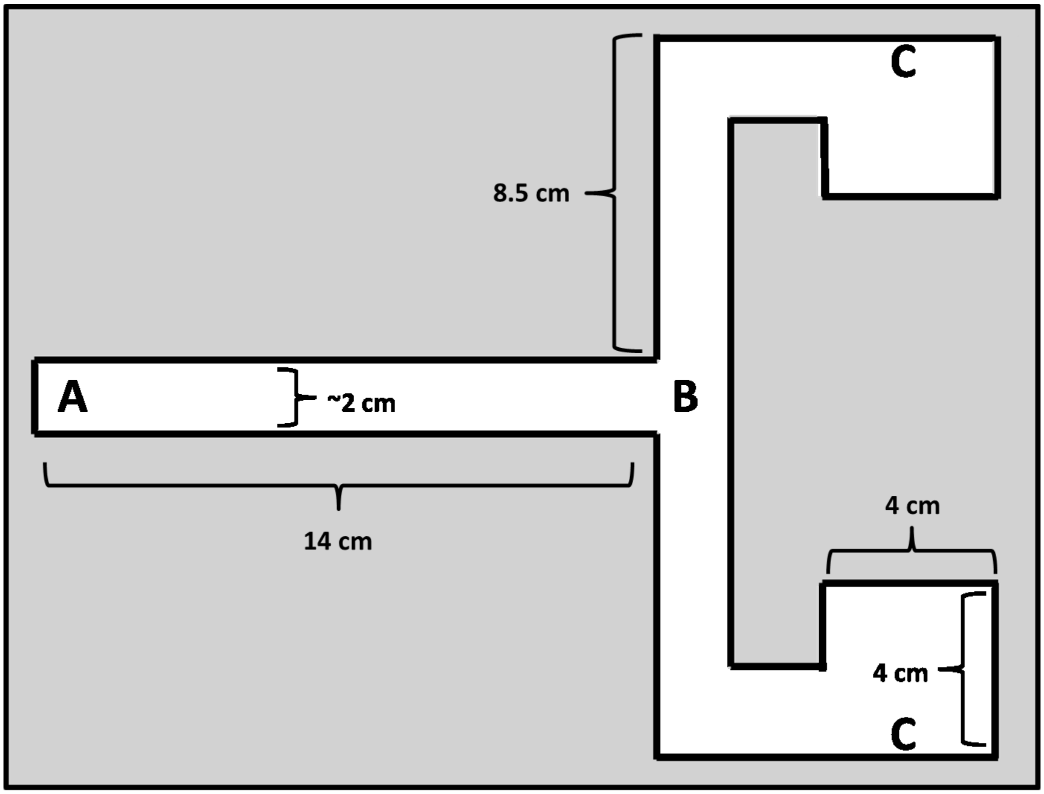

Figure 2.1. Y-maze apparatus used in male inhibition trail following studies. A) release point, B) first turn choice, C) choice end chamber.

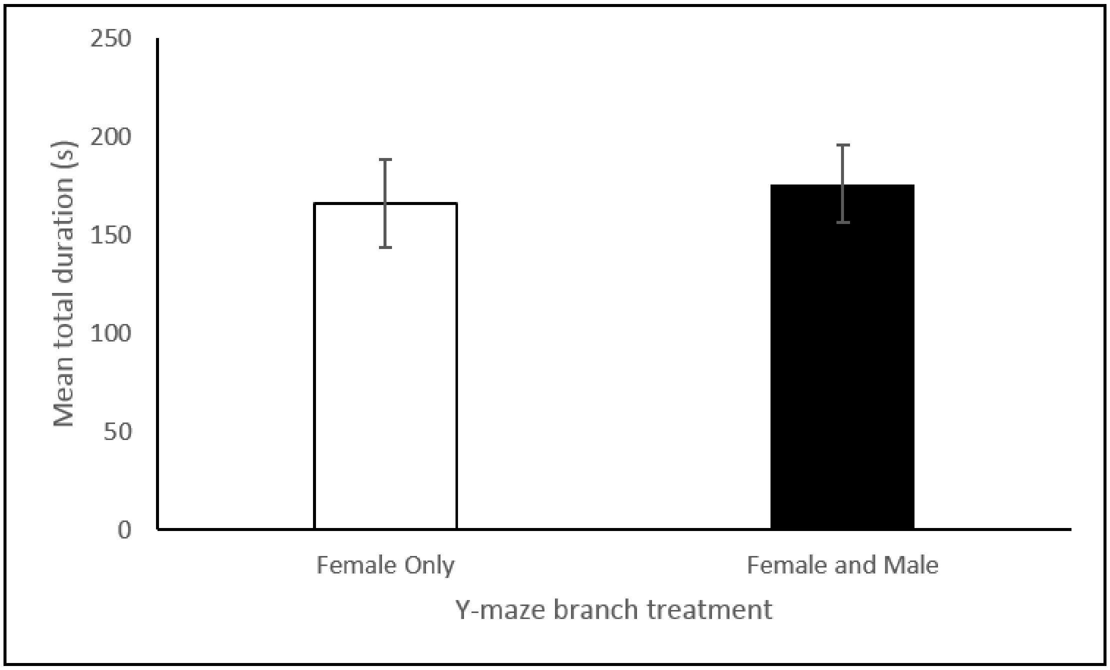

Figure 2.2. Mean total duration (s) (±SE) of time spent in the treated branches of the Y-maze.

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Chapter 3: Trail Following in *Schizocosa ocreata*

ABSTRACT

Male spiders are able to detect and respond to female chemical cues deposited with the silk of females in the environment. In many species, detection of these chemicals may be the first indication a male has to the presence of a nearby female. We used behavioral assays to determine whether male *Schizocosa ocreata* (Hentz 1844) wolf spiders (Lycosidae) are able to detect and respond to cues resulting from a single pass trail by a female, and whether they are able to determine the direction of female travel. Our focal males responded to virgin adult female trails with following behavior, but showed no propensity to follow trails from other conspecifics (subadult females or males). While they were able to follow a female trail, our analysis indicates that they are not able to determine trail directionality.

INTRODUCTION

Among the primary signaling modalities (visual, vibrational, chemical), chemical signaling is the most taxonomically widespread (Greenfield 2002; Wyatt 2003; Thiel & Breithaupt 2011), and likely represents the earliest form of communication (Regnier & Law 1968; Wyatt 2003; Bradbury & Vehrencamp 2011). The ability to perceive and respond appropriately to chemical cues in the surrounding environment plays an important role in the behavior and ecology of many animal species (Wyatt 2003; Bradbury & Vehrencamp 2011). Detection of heterospecific chemical cues may be important in locating prey or host plants (Jones 1986; Phelan et al. 1991; Bénédet et al. 2002; Helms et al. 2003), and avoiding predators (Lima & Dill 1990; Kats & Dill 1998; Dicke & Grostal 2001; Persons & Rypstra 2001).

Conspecific signals can convey information relating to many different social contexts (Wyatt 2003), including aggregation (Al Abassi et al. 1998), territory marking and social dominance (Perret 1992; Moore et al. 1997), identification of individuals and groups (Greenfield 2002), and alarm (Wyatt 2003). Conspecific chemical signals can also encode information pertaining to age, sex, and mating status, any of which could be critical to determine if another individual is a suitable mate (Jacobson 1972; Wyatt 2003; Schulz 2004).

In studies of taxa utilizing chemical information, the production and detection of chemicals is generally subdivided into airborne (olfactory) and tactile (contact, including gustatory) components. These components are under very different selection regimes, and are typically different classes of chemicals as a result. Chemicals that operate in olfaction must be small (usually less than 300 molecular weight), and volatile, meaning they are highly dispersible in air (Bossert & Wilson 1963; Regnier & Law 1968; Okubo & Levin 2001). Olfaction can occur at a range of distances between source and receiver based on quantity of compound produced, the detection threshold of the receiver, and the rate at which the component moves through the environment via diffusion and bulk air movement (Bossert & Wilson 1963; Regnier & Law 1968).

In contrast, contact chemoreception requires direct contact between the receiver and a cue source (Wyatt 2003; Bradbury & Vehrencamp 2011), which may be the sender itself, or cues that have been excreted or secreted in the environment. Compounds involved in contact chemoreception are typically of a much larger molecular weight and thus are non-volatile (or nearly so), and the chemical classes utilized may be quite broad, including long chain hydrocarbons, fatty acids, lipids, or proteins (Pawlik & Faulkner 1986; Mason et al. 1989; Johnston 2003; Schal et al. 2003, El-Sayed 2014). Not only do these compounds have limited dispersal in the environment, they may remain stable and accessible to receivers long after the sender has left the area (Bradbury & Vehrencamp 2011), potentially resulting in a temporal disconnect between the receiver's behavior and the context in which it would be appropriate.

The use of contact chemoreception is especially widespread and well-studied among arthropods. In crustaceans, contact chemoreception has been implicated in mate finding and larval settling (Pawlik & Faulkner 1986; Frey et al. 1998; Bauer 2011; Hay

2011; Snell 2011), and plays an important role in mediating hexapod host and oviposition site finding (Baur et al. 1998; Chapman 2003; Newland & Yates 2008), social aggregation (Roessingh et al. 1988), and identification and location of a suitable mate (Schal et al. 2003; Rutledge et al. 2009; Thistle et al. 2012).

Within arachnids, and spiders in particular, the use of contact chemical communication is thought to be widespread and an important aspect of intersexual signaling, but is comparatively unexplored relative to other arthropod systems. The majority of studies looking at contact chemoreception among spiders have focused within the context of intersexual signaling (Schulz 2004; Roberts & Uetz 2005; Gaskett 2007; Uhl & Elias 2011; Schulz 2013), which generally occurs from female signalers to male receivers. Contact chemoreception of biologically active compounds occurs via tip pore sensilla located on the legs and pedipalps (Foelix & Chu-Wang 1973; Kronestedt 1979; Tietjen & Rovner 1982; Tichy et al. 2001). These chemosensory "hairs", when contacting the silk and/or cuticle of a female, pick up signals that can elicit male courtship behaviors (Schulz & Toft 1993; Prouvost et al. 1999; Papke et al. 2000; Tichy et al. 2001; Trabalon et al. 2005; Jerhot et al. 2010).

The brush-legged wolf spider, *Schizocosa ocreata*, is particularly well suited to exploring questions pertaining to the use of signaling modalities and has been studied extensively in the context of multimodal signaling and mate choice (Hebets & Uetz 1999; Uetz & Roberts 2002; Hebets & Papaj 2005; Uetz et al. 2009; Hebets 2011). *Schizocosa ocreata* typically inhabits a spatially and structurally complex deciduous leaf litter environment (Uetz 1979; Cady 1983; Dondale & Redner 1990), and habitat complexity likely results in unreliable signaling in any given modality at any particular time (Grafe et al. 2012; Uetz et al. 2013). This, in turn, favors the evolution of a complex sexual communication system using multiple modalities simultaneously (Hebets 2011). It is well established that *S. ocreata* individuals utilize different modalities when information in any particular modality is compromised or unavailable (Taylor et al. 2006; Gibson & Uetz 2008; Gordon & Uetz 2011; Uetz et al. 2013).

Upon encountering a female, or even female chemical cues alone (likely via silk dragline), a male will engage in a vigorous courtship display simultaneously broadcasting visual and seismic components, including tapping and waving of tufted forelegs, stridulation, and bouncing of his body/chelicerae against the substrate (Bristowe & Locket 1926; Uetz & Denterlein 1979; Stratton & Uetz 1981; Stratton & Uetz 1983; Hebets & Uetz 1999). A male displaying in this manner in the presence of chemical cues, but absence of a female, will incur high energetic costs (Cady et al. 2011), as well as increased detection by predators (Pruden & Uetz 2004; Roberts et al. 2006; Hoefler et al. 2008; Fowler-Finn & Hebets 2011a,b). However, as a species exhibiting a scramble competition mating system it may be a selective advantage for polygynous males to engage in display even if they cannot be certain that a monandrous females will receive his display (Norton & Uetz 2005), just for the chance at mating priority. Within the habitat, *S. ocreata* may be patchily distributed and can occur in relatively high density within suitable microhabitats (Aspey 1976; Cady 1983), so even if the female which deposited cues is no longer present, his display may be detected by other nearby (but hidden) females, increasing the potential advantage of a risky, expensive display. In addition to increasing the likelihood of successful copulation, active courtship by a male decreases his risk of becoming prey for aggressive but hidden females (Delaney et al. 2007; Gibson & Uetz 2008).

Surprisingly, despite their apparent widespread use, relatively little is known about the identity and utility of chemical cues in this otherwise well studied model system. Lycosid spiders produce a silk dragline as they move through the environment (Richter 1970; Richter et al. 1971; Tietjen 1977), so substrate bound cues available to the male would include any compounds present on the silk or deposited by contact of the female cuticle with the substrate. Many studies looking at male response to female chemical cues utilized substrates upon which the female had made many passes, or resided for a substantial amount of time, often as much as 12 hrs (Uetz & Denterlein 1979; Stratton & Uetz 1986; Roberts & Uetz 2004a,b; 2005). While areas of particularly high cue density may be encountered in the wild (Aspey 1976), they likely represent a more concentrated stimulus than what is most commonly encountered, as field conditions can rapidly degrade the chemical signal (Baruffaldi et al. 2010; Roberts unpubl.).

The capacity for a male to detect a single female trail is likely under strong selection pressure, as it could provide him with information on the recent passage and proximity of a potential mate. If males are only able to detect relatively high concentrations of female cues, as may be associated with an area a female has resided in for an extended period, his ability to find a mate would be contingent on randomly encountering these patches of high cue density. If instead he is able to detect and follow a female’s unidirectional, single-pass trail, it may lead him directly to her, or at least increase his likelihood of encountering other cues that inform his behavior.

If males do demonstrate the ability to recognize and follow female trails, the ability to follow female cues in the direction which they were deposited would provide the greatest increase in likelihood of encounter with the female that produced the cue trail. Directional following has been demonstrated in wolf spiders, but not conclusively in *S. ocreata* (Tietjen 1977). In the current study, we tested the hypothesis that male *S. ocreata* will be able to detect female cues deposited on a substrate in a single pass, and will respond to female trails with following behavior.

METHODS

Spider collection and rearing

We collected immature *S. ocreata* spiders at The Dawes Arboretum, Newark, Ohio, USA (N 39.973863, W -82.40128) during April and October 2012, and raised them in the lab for use in trials. All of the spiders we used were housed in individual plastic containers (500ml, 9cm diameter, round) on a substrate of moist coconut fiber which provided water *ad libitum*. We fed spiders 2-3 *Acheta domestica* cricket nymphs twice weekly, and maintained them on a 13:11 h light:dark cycle at room temperature (22-25°C).

Experiment 1- Single pass trail following

In order to evaluate male *S. ocreata* response to conspecific cues deposited on the substrate, we used a Y-maze style choice test. We used a modified open-bottom Y-maze design (Fig 3.1), assembled on a vibrationally isolated marble slab and covered with Plexiglas to prevent spider escape while allowing for video recording of each trial. We designed the central leg and choice arms to be of narrow width (approx. 2 cm), which is less than the average *S. ocreata* leg span of approx. 3.0cm (Aspey 1976), to reduce thigmotactic 'edge following' effects by the focal spider. We placed a fresh sheet of white paper on the marble slab below the maze for each trial to serve as a substrate.

We assessed male trail-following response based on cue trails from female (N=32), male (N=21), or subadult (N=24) conspecifics. To prepare for a trial, we randomly blocked one arm of the maze, allowing cues to be deposited in the other arm only. Adult spiders used were 1-4 weeks post final molt, and subadult females were 1-2 instars from molting to maturity. We randomly selected a stimulus spider (female, male, or subadult female, as appropriate), placed the stimulus animal at the start of the maze, and gently guided it (in a single pass) through the maze and into the end chamber before removing it from the apparatus. This procedure resulted in a uni-directional cue trail through the apparatus, as well as a blank path to the opposite choice chamber. A randomly selected focal male (1-4 weeks post adult molt) from the lab population was introduced at the start of the maze and we video recorded his behavior for ten minutes (Canon VIXIA HF S100 HD video camera).

When presented with trails established by females, males or subadults in a Y-maze, we scored focal male choices as either following or not following the trail established by the stimulus individual at the first turn made and the first end chamber entered, and used a Chi-square test with the assumption of no difference between following or not following. Using an ANOVA design, we also examined log transformed latency of focal males to make first turn and end chamber choices in the Y-maze, as well as total time spent in either choice arm. Males that did not move beyond the initial leg of the maze were excluded from analysis.

After each trial, we removed the paper substrate and wiped down all surfaces of the apparatus, marble, and Plexiglas with 99% ethanol and a Kimwipe to remove all traces of chemical and silk cues from previous stimulus and test individuals. We set the apparatus aside and allowed it to air dry before preparing the next trial.

Experiment 2 - Directional female trail following

To evaluate whether male *S. ocreata* are able to determine the direction a female conspecific was moving, based on her trail alone, we used the same maze apparatus described previously (Fig. 3.1), but modified the pattern of female silk deposition. Prior to the start of each trial we placed a fresh piece of white paper under the maze to serve as a substrate, and blocked off the central leg of the maze. We placed a randomly selected female (2-6 weeks post adult molt) in either the left or the right end chamber and gently guided her down the arm, past the blocked central leg, and up the other arm and into the other end chamber, where we removed her from the apparatus. This resulted in a uni-directional female trail from one arm to the other, with direction the female would be passed through the Y-maze (right to left, left to right) determined randomly before each trial.

We introduced a randomly selected focal male (2-7 weeks post adult molt) (N=29) at the start of the maze and recorded his behavior over a ten minute trial. A male moving up the central leg would thus encounter a female trail we established perpendicular to his movement, and he could either move in the same direction as the female (following), or the opposite direction from the female (not following). We scored focal male choice based on which direction they first turned, as well as the first end chamber they reached. Our statistical analysis using Chi-square ($\alpha = 0.05$) assumed no difference between following or not following the trail in the correct direction.

RESULTS

Experiment 1 - Single pass trail following

Our Chi-square analysis revealed a significant effect of female cues on male path choice, with males much more likely to make a first turn to follow a female trail, and no propensity to follow or not follow cues associated with a male or subadult trail (Table 3.1). Because focal males may make a 'false start' down one branch before reversing direction and moving down the other, we also looked at the first end chamber entered by each male, again using Chi-square analysis under an assumption of no difference. We observed the same pattern of males being more likely to follow female trails to the termination of the maze, and subadult and male trails having no significant difference (Table 3.1).

To examine whether cue source influenced the rate of movement through the maze, we examined latency of males to make their first turn (Fig. 3.2.A), but found no significant difference across treatments (ANOVA, $F_{2,75} = 0.52$, $p = 0.60$). We also examined latency for males to enter their first end chamber (Fig. 3.2.B), but again found no significant difference between treatments (ANOVA, $F_{2,75} = 0.051$, $p = 0.95$).

Since males were permitted to move freely through the maze during the trial, even after making first turn and end chamber choices, we observed many males moving back and forth between both arms of the maze, as well as back up the initial leg. We scored the mean duration of time spent in the cue treated branch of the maze, but did not find any significance across treatments (ANOVA, $F_{2,75} = 0.27$, $p = 0.7628$). Since the initial leg also contained treatment cues, we compared the amount of time spent on the cue trail (initial leg plus treated arm) across treatments, and although there was a tendency for focal males to spend more time on female cues, was not statistically significant (ANOVA, $F_{2,75} = 2.54$, $p = 0.0859$).

Experiment 2 - Directional female trail following

We scored male *S. ocreata* as having correctly followed a female trail upon turning or entering the end chamber in the direction the female trail was established. Focal males did not show a significant tendency to follow female cues in the direction of her travel, with only slightly over half of the males tested making their first turn in the correct direction ($X^2_{1,29} = 0.86$, $p = 0.35$), and slightly less than half arriving at the end chamber corresponding to the correct direction of travel ($X^2_{1,29} = 0.31$, $p = 0.58$).

DISCUSSION

Mate finding is an important evolutionary consideration for many species, especially in those organisms where copulation is a necessary component of reproduction. In the forest leaf litter dwelling wolf spider, *Schizocosa ocreata*, the more active males move throughout the environment in search of much less active females (Cady 1983). Among the signaling modalities utilized by the species, a wandering male may often be constrained in terms of which modalities he is able to use to detect the presence of a nearby female. If she remains motionless and buried within the leaf litter, she may be visually isolated and generating no seismic signals that he can detect. Often chemical cues associate with her passage through the environment will be the first indication a male has of a female's presence. We would expect that being able to detect minute levels of cues associated with a potential mate, and use that information to aid in locating her, would be strongly selected for in this species.

In the single pass trail following experiment, male *S. ocreata* engaged in following behavior (Table 3.1), demonstrating the ability to detect a single-pass trail from females and subsequently follow it through the follow it through the Y-maze. Following behavior was absent in trials where the cues were generated by subadult and male conspecifics, indicating that males are likely responding to a chemical component deposited in or on female silk or by female cuticular contact with the substrate. Although the presence of silk may provide additional physical cues, males only demonstrated following behavior on female trails. Silk may indicate the passage of a spider, but chemical cues likely provide the basis of distinguishing sex and maturity. Trail following behavior has not been established previously in *S. ocreata*, and was suggested not to exist in at least one previous study (Tietjen 1979). While male *S. ocreata* do not display the palpal cradling of the female dragline observed in other lycosid species (Tietjen 1977), the work presented here does suggest they have some capacity to follow female cues.

Focal males were not faster to make first turn or end chamber choices in the presence of female cues, nor did they spend a significantly greater proportion of time during the trial in the presence of female cues. This suggests that the following behavior indicated by male movement in the female cue trail trials is not due simply to males spending more time in association with female cues. However, the time spent may be affected by the design of the maze itself. After following the maze to its terminus and failing to find a female, the focal male may have a tendency to explore other parts of the maze rather than remain only in the areas containing female cues.

It should also be noted that while males did not follow either subadult or male trails, neither did they strongly avoid them. Ayyagari and Tietjen (1986) demonstrated the cues associated with extracts from male silk to have an inhibitory effect on male behaviors, but the current study shows no tendency to avoid cues associated with the trails of potential competitors.

Our study looking at directional female trails suggests that males are not able to glean directional information from female cue trails. This may be a reflection of *S. ocreata* not engaging the draglines with their palps (Tietjen 1977), which would decrease the amount of physical dragline structure that they would be able to detect and utilize to determine direction. There may be little or no selection for strict directional following in a system where male encounters with females are relatively frequent (Aspey 1976), and strict adherence to following it may result in missing other courtship and mating opportunities.

In summary, we found that male *S. ocreata* are able to detect cues associated with the trails of female conspecifics, and follow even a single pass trail through a Y-maze. A single pass trail represents the minimum amount of chemical information that a male may encounter in the environment, suggesting that the chemosensory system involved in detection of female trail associated cues is highly sensitive. As expected, males did not show a tendency to follow trails established by subadults or other males, neither of which would represent a viable mating opportunity, nor did they show avoidance of cues associated with these other conspecifics.

The lack of directional discrimination by males suggests that if they do encounter a female trail in nature, they are no more likely to follow it in a direction that would lead them to that female than they are to follow it in the opposite direction. Males that are unable to determine the correct direction in which to follow a female trail would not be expected to hold to a strict trail following behavior, but may instead search for additional female cues in close proximity to a detected trail, which would increase the likelihood that they may encounter additional females or female cues.

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Trail Cue Treatment	First Turn Follow				End Chamber Follow			
	Cue	No Cue	Chi-square	p-value	Cue	No Cue	Chi-square	p-value
Subadult Female	12	11	0.043	0.83	12	11	0.043	0.83
Adult Male	9	12	0.43	0.51	9	12	0.43	0.51
Adult Female	25	7	10.13	0.0015*	22	10	4.5	0.034*

Table 3.1. Results of Y-maze single pass trail following by male *Schizocosa ocreata*. Males showed a significant tendency to follow female cue trails only (*).

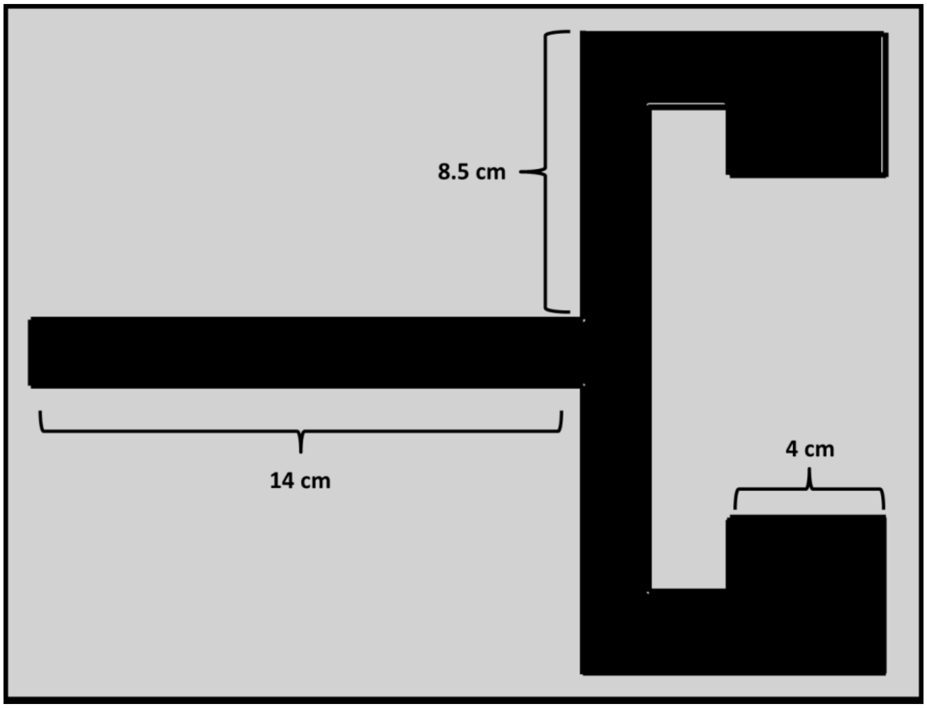

Figure 3.1. Y-maze apparatus used in trail following studies. A) Release point, B) First turn choice, C) Choice end chambers.

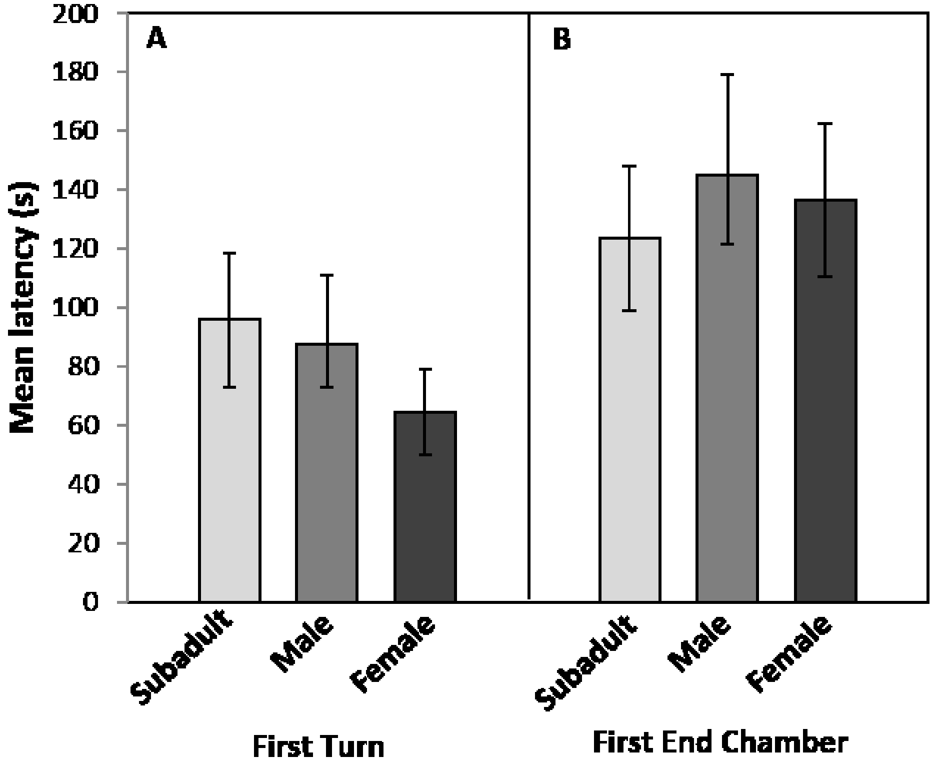

Figure 3.2. Mean latency (s) (±SE) from the start of the trial for focal males to make a choice A) of first turn, and B) end chamber.

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Chapter 4: *Schizocosa ocreata* silk collection, extraction and analysis

ABSTRACT

Identification of spider sex pheromones has lagged behind other arthropod groups, despite behavior in the context of chemical communication being widely studied in many species. As they move around the environment, female wolf spiders deposit silk draglines along with substrate bound sex pheromones. Reception of these pheromones can elicit male courtship behavior, but the activity of these pheromones is rapidly degraded by exposure to polar solvents. In this study we developed a means by which to collect silk dragline and associated chemical cues from female *Schizocosa ocreata* (Hentz 1844) for extraction and analysis. Analysis revealed four female specific compounds, and presentation of female silk extract to male spiders elicited strong chemoexploratory behavior, but not courtship displays.

INTRODUCTION

Communication involves the production of a signal by one individual, its transmission through the environment and its reception, neural processing, and integration by a second individual, resulting in a physiological or behavioral modification in the receiver. These signals may be visual, vibratory (seismic or acoustic), or chemical in form. Chemical signaling involves the production of chemicals that comprise an informative signal, and likely represent the oldest method of communication (Wyatt 2003). Chemical compounds involved in communication are collectively referred to as semiochemicals, and are divided between interspecific (allelochemicals) and intraspecific communication (pheromones) (Law & Regier 1971).

Sex pheromones are taxonomically widespread and highly diverse, constituting a wide range of organic compounds of varying size, functional groups and bonding complexity (Roelofs et al. 2002; Symonds & Elgar 2008; El-Sayed 2014). However, there is strong potential for convergence or conservation of pheromones across disparate mating systems (El-Sayed 2014), as shown in the work of Rasmussen et al. (1996), who identified a pheromone shared between Asian elephants (*Elphas maximus*) and over a hundred species of moths. Chemical cues play an important role in arthropod communication (Greenfield 2002), especially where conspecifics can be widely separated, cryptic within their habitat, and as a result, isolated from one another in other modes of communication.

The role of arthropod sex pheromones in intraspecific communication has been most widely studied in insects (Jacobson 1972; Phelan et al. 1986; Mori 1989; Phelan 1992; Franke & Dettner 2005; Millar 2005; Symonds & Elgar 2008), and crustaceans (Hardege & Terschak 2011; Hay 2011) with comparatively few investigations and pheromone identifications among spiders (for review see Schulz 2013). The chemical modality has long been known to play a role in spider communication (Bristowe & Locket 1926; Kaston 1936), but the majority of spider chemical communication studies have only behaviorally demonstrated the presence of active compounds (for review see Tietjen & Rovner 1982; Schulz 2004; Foelix 2011), with little or no attempt to identify them.

Biologically active compounds may be produced as secretions from glands and released or bound on the individual's integument, or emanate from digestive or reproductive orifices (Bradbury & Vehrencamp 2011). The production of silk from abdominal spinnerets is unique to spiders, and spider silk has been shown to be an important medium through which pheromones can be introduced to the environment (Dondale & Hegdekar 1973; Jackson 1986; Gasket 2007). The first spider pheromone was structurally identified relatively recently from extracts of silk (Schulz & Toft 1993), and pheromones have only been identified in five additional families of spider since (Schulz 2013). Within the limited number of spider species from which a sex pheromone has been identified, a few have been isolated from the cuticle alone (Papke et al. 2001), but the majority have been extracted from spider silk (Schulz & Toft 1993; Tichy et al. 2001; Xiao et al. 2009; Chinta et al. 2010; Jerhot et al. 2010).

In order to study silk associated compounds, clean silk samples must be collected, but the ease of collecting silk for extraction and analysis varies based on species and the ecological function of the silk within the spider family to which the species belongs. For species that build them, webs serve as a readily collected source of relatively uncontaminated silk (Schulz & Toft 1993; Xiao et al. 2009; Chinta et al. 2010; Jerhot et al. 2010). For spiders that do not build a web, this is not possible, and silk must be collected by other methods. For some web and non-web building spiders, forced silking by restraining the spider and drawing silk out onto a motorized reel may be a viable means of collecting uncontaminated silk (Work 1976; Papke et al. 2000; Tichy et al. 2001; Blackledge et al. 2005). Draglines deposited on a substrate can be collected for use (Baruffaldi et al. 2010), but while maintaining spiders on a substrate for a longer period of time will likely increase the amount of silk deposited, it also creates a greater potential for contamination by non-silk compounds (fecal, cuticular, substrate).

Schizocosa ocreata is a leaf litter dwelling species of wolf spider common in eastern deciduous forests of the United States, and utilized as a model system for the study of sexual selection and multimodal communication (Hebets & Uetz 1999; Uetz 2000; Uetz & Roberts 2002; Hebets & Papaj 2005; Stratton 2005; Hebets et al. 2011). Males of the species are highly attuned to female chemical cues deposited with the silk dragline as she moves through the environment, engaging in visual and seismic species specific courtship displays upon encountering evidence of the presence of a female (Bristowe & Locket 1926; Uetz & Denterlein 1979; Stratton & Uetz 1981; Stratton & Uetz 1983; Hebets & Uetz 1999). The leaf litter habitat where this species is found can have a high level of structural complexity (Uetz 1979; Cady 1983), which may prevent visual or vibrational detection of a potential mate (Scheffer et al. 1996; Elias et al. 2006; Gordon & Uetz 2011). Detection of female chemicals deposited in the environment may be a male's first indication that a female may be nearby.

As with many spider species, while chemical communication in the wolf spider *Schizocosa ocreata* has been demonstrated behaviorally (Tietjen 1979; Uetz & Denterlein 1979; Tietjen & Rovner 1982; Roberts & Uetz 2004a,b, 2005; Gaskett 2007), no pheromones have been fully extracted and identified (Ayyagari & Tietjen 1986). Extraction of female silk with a polar solvent has been shown to reduce or eliminate male courtship response to the washed silk once allowed to dry in both *S. ocreata* (Roberts & Uetz 2004a), and in *S. malitiosa* (Baruffaldi et al. 2010). However, while dosage of the silk extracts on fresh substrate restored male response in *S. malitiosa* (Baruffaldi et al. 2010), it did not in *S. ocreata* (Roberts & Uetz 2004a). Whether this is due simply to differences in the chemistry of the active compounds, or variations in the concentration of the stimulus or detection threshold is not clear at this time. The collection of relatively uncontaminated silk is a prerequisite to further chemical analysis of *S. ocreata* substrate bound pheromones. However, attempts to harvest silk through forced silking have been unsuccessful, as restrained spiders will break the dragline before an appreciable quantity of silk has been collected (Roberts pers. obs.).

Understanding the chemical composition of *S. ocreata* pheromones may aid in resolving phylogenetic relationships between this species and other members of the genus. In the genus *Schizocosa*, a recent morphological phylogeny resulted in a paraphyletic group comprised of three populations of *S. ocreata* (from OH, MS and FL) within a distinct clade including five other *Schizocosa* species (Stratton 2005). Identification of the chemical structure of compounds involved in mate finding and eliciting courtship could potentially add another character to the phylogeny, and aid in further refining our understanding of *Schizocosa* evolutionary relationships. Additionally, male *S. ocreata* respond strongly with courtship behavior to heterospecific female cues in the case of *S. rovneri*, at a reduced level to cues of female *S. crassipes*, and a further reduction to cues from the more distantly related *S. saltatrix* (Roberts & Uetz 2004b). This suggests a varying level of conservation of female pheromones within this clade and genus, which could be an important character in understanding the recent diversification of these species.

In the current study, focusing on chemical analysis of *S. ocreata* silk, we developed a turntable apparatus suited to *S. ocreata* silk deposition behavior and used it to collect low contaminant silk samples. After extraction and analysis, we used the remaining solvent cue extracts to assess male response to treated filter paper in an attempt to reestablish normal male behavioral response to extracted female compounds.

METHODS

Spider collection and rearing

We collected *S. ocreata* spiders as immature individuals from The Dawes Arboretum, Newark, Ohio, USA (N 39.973863, W -82.40128) during October 2014 and from private land in Fresno, Ohio, USA (N 40.339033, W -81.757291) in April 2015. We raised them to maturity in the lab until used for silk collection. We housed all spiders on moist coconut fiber substrate in individual plastic containers (500ml, 9cm diameter, round). The substrate provided water *ad libitum*, and we fed the 2014 spiders 2-3 cricket nymphs (*Gryllodes sigillatus*) twice weekly, while the 2015 spiders were fed a mixed diet of *D. melanogaster*, *D. hydei* and *G. sigillatus*. We maintained lab populations at room temperature (22-25°C) on a 13:11 h light:dark photoperiod.

Cue collection

We utilized an apparatus that keeps spiders stationary while the substrate moves beneath them to collect silk samples. Our goal was to allow the spiders to walk approximately normally, while laying down dragline silk and chemical cues we could collect for chemical extraction. We kept spiders restrained during the collection procedure to ensure cues were deposited in a concentrated area, and also to prevent their escape. To achieve this, we utilized a procedure modified from Long et al. (2015) to affix each spider to a dental microbrush (Azdent MA-101 Fine) with melted dental wax (base plate wax, pink) using a dental wax carver (Model SJK-110), which allowed us to precisely set implement temperatures and limit excessive thermal stress to the spiders. To prepare the microbrush, we removed the tip and attached a small spring to the shaft, which allowed us to reattach the microbrush tip to the other end of the spring (Fig. 4.1). This resulted in a flexible attachment shaft which was more forgiving of spider movements and more likely to allow them to deposit cues on the substrate. We secured the spring to both sections of microbrush with melted wax, and replaced the tip with a fresh one for each spider.

We followed the procedure of Long et al. (2015) to restrain spiders for waxing by removing the end from a 30mL syringe and retracting the plunger. We placed the spider within the syringe barrel and stretched a small square of Parafilm over the top. When we slowly depressed the plunger, it brought the spider up against the Parafilm barrier, and we used the tip of a metal probe to gently tear a hole in the Parafilm above the cephalothorax. In order to attach the spider, we loaded the fine bristled tip of the microbrush with a droplet of wax, which we then held in contact with the cephalothorax of the spider and using a narrow wax carving tip, melted the wax to the spider. Once the wax solidified, we removed the Parafilm constraint and the spider was allowed a 5 min rest period before cue collection.

The silk collection apparatus consisted of a circular turntable (30 cm dia) with a clean disk of filter paper (Fisherbrand, 9 cm dia) in a glass Petri dish (Kimex, 9 cm dia) placed in the center, on which we lowered our restrained spiders (Fig 4.2). The use of a variable-voltage DC power supply (Mastech HY3005F-3) and a gear reduced electric motor allowed us to adjust the rotational speed of the turntable, and thus the walking speed of the spider. This allowed us to set the speed of the turntable to a speed at which the spiders were able to walk in an approximately normal manner (~1 rpm).

Prior to use, we cleaned the Petri dish with detergent and tap water, then triple rinsed with distilled water, HPLC grade water, and HPLC methanol before allowing it to dry. Our manipulation of filter paper was done with clean forceps that were rinsed in HPLC grade methanol between each sample. We secured the filter paper to the Petri dish with melted wax in the center to prevent it from rotating and clamped the shaft in place to keep the spider in close contact with the filter paper, at an appropriate distance where they would be able to reach the paper with their spinnerets, but not be dragged across its surface. We positioned each spider 1cm from the edge of the filter paper, to limit their ability to grasp the edge and to ensure that all cues being deposited were on the filter paper and not on the Petri dish.

To start the collection of cues, we activated the turntable for 2 minutes, after which it was turned off for 30 seconds to allow the spider a period of rest. We continued this for thirty rounds of on and off, totaling 60 minutes of walking time per collection. Where we positioned the spider on the filter paper, each collection spider traveled approximately 13.2 m (22 cm per rotation, 60 minutes at 1 rpm). At the end of the collection period, we removed each spider and detached it from the microbrush tip. Using clean, methanol rinsed forceps and scalpel, we excised the center of the filter paper disk where it had been waxed to the Petri dish. This left a circular ring of filter paper that encompassed the path walked by the spider. This ring was cut into quadrants before being carefully rolled and inserted into an 8mL sterile borosilicate glass vial for storage at -80°C. We collected silk from adult male (N=3), adult female (N=3), subadult male (N=3), and subadult female (N=3) spiders. Adult spiders used were 1-2 weeks post maturity, and subadults were within 1-2 molts of maturity. We also collected control samples consisting of blank filter paper that was waxed to the Petri dish, excised and cut into quadrants, and stored at -80°C in the same manner as samples containing cues.

Chemical analysis

We submitted samples to the Campus Chemical Instrument Center at The Ohio State University for analysis. Filter paper samples were pooled by sex and age to increase the likelihood of detecting compounds of interest. Samples were cut into small fragments with clean scissors to allow for increased agitation and movement of the extracting solvents across the sample. Methanol was chosen as the solvent used for extraction, as it has been previously show to be highly effective in reducing male response to female silk following solvent extraction (Roberts & Uetz 2004a). After adding the methanol to the filter paper fragments, the samples were alternately vortexed and sonicated for four hours to aid in extraction efficiency. The resulting solvent was drawn off and concentrated under nitrogen evaporation, then stored at -80°C until analysis via gas chromatography - mass spectrometry (GCMS).

Male response to concentrated silk extract

We utilized the nitrogen evaporated samples left over after GCMS analysis as silk extract stimuli to determine if more highly concentrated extracts results in male courtship response to female cues. Because volumes and samples were limited, we pooled concentrated samples to create three treatment stimuli; 1)male extract (N=6), 2) female extract (N=5), and 3) filter paper blank extract (N=5). To treat a disk of filter paper, we pipetted 5µL of the extract onto the disk, 1 µL at a time, allowing it to completely air dry after each application. We ran trials on a vibrationally isolated marble slab in an arena enclosed in a Plexiglas ring (10 cm dia), where all surfaces had been wiped with 99% ethanol to remove any silk or chemical cues, and allowed to dry. To begin each trial, we placed the treated disk of filter paper in the arena before introducing the male (1-4 weeks post maturity), and video recorded (Canon VIXIA HF S100 HD video camera) focal male behavior during a five minute trial, after which we scored movement and courtship behaviors using J-Watcher (V1.0)

RESULTS

Chemical analysis and male response to concentrated silk extract.

Our GCMS results identified four compounds only present in female treated filter paper extracts (Fig. 4.3). The response of our focal males to filter paper treated with these extracts showed a statistically significant difference only in the mean duration of chemoexploratory behavior (ANOVA, $F_{2,16} = 33.0$, p-value = <0.0001) (Table 4.1). Pairwise comparison revealed a significant difference between male chemoexploratory behavior in the presence of female derived extracts versus extracts from males or blank filter paper, and no difference when comparing male to blank (Fig. 4.4). Furthermore, focal male chemoexploration on female treated filter paper was observed to take place directly over where the extract had been applied.

DISCUSSION

Our ability to collect silk in sufficient quantities with as little contamination as possible was an important consideration in improving the potential for a successful chemical analysis. We were concerned that leaving spiders on filter paper for multiple hours or overnight would increase the chance of contamination. Fecal excretions absorb readily into filter paper, and can be excised, but even if we did so, there would be the potential for fecal contamination to be tracked across the substrate. Since *S. ocreata* are not amenable to forced silking, we wanted to devise a way to allow them to walk and deposit dragline as normally as possible. Our variable speed turntable functioned as desired, and we observed spiders touching their spinnerets to the filter paper in a manner consistent with establishing an attachment disk, which is a necessary component of leaving a dragline.

Our focal males exposed to female extract showed a significantly greater chemoexploratory response compared to males exposed to either male or blank extracts (Fig. 4.4). Not only did they spend more time chemoexploring, but they engaged in this searching behavior only in the location where the extract had been applied. Any chemoexploration by focal males in the other two treatments did not take place at the treatment location. Males are responding to something present in the female extracts, potentially one of the female specific compounds identified in our chemical analysis (Fig. 4.3). Our sample size was quite limited due to the low volume of concentrated extract available, dilution with methanol or application of a smaller volume to the filter paper may have allowed for a greater sample size, but would limit the quantity of compound present, potentially reducing it below the detectable threshold.

We did not observe any difference in jerky tapping, a courtship display behavior released in the presence of female silk. While extracts of female silk did elicit a strong chemoexploratory response, some component of the female signal responsible for eliciting courtship is lacking (Table 4.1). It is possible that there are multiple chemical components functioning in this system, and a female compound eliciting chemoexploration was able to be extracted, while the compounds impacting courtship were not. Roberts & Uetz (2004a) found that a methanol wash of silk did not eliminate chemoexploratory response to the silk, but did not observe a strong chemoexploratory response to the extract itself. It may also be possible that some other aspect is necessary, such as physical cues from silk being present. The four compounds identified (Fig. 4.3) would be a likely place to begin testing male response to determine if one (or more) increases male chemoexploration.

Of the female specific compounds identified, octadecanoic (stearic) acid, is the most widespread across taxa, and has been identified in chemical studies of mites, insects, mammals, and reptiles (El-Sayed 2014). This saturated fatty acid was found by Trabalon et al. (1997) in silk extracts of the Agelenid spiders *Tegenaria domestica* and *T. pagana*, but it was determined not to be the attractive component. Stearic acid is also a metabolic precursor to one of the pheromones identified in the Pholcid spider *Pholcus beijingensis* (Schulz 2013).

The lack of male courtship behavior (jerky tapping) in response to the extract of female silk (Table 4.1) suggests that the chemical(s) responsible for eliciting this behaviors is not among the female specific compounds identified (Fig 4.3). Additionally, cyclohexyl isothiocyanate, oleonitrile, and stearic acid are all fairly insoluble in water (solubility data was not available for N-methoxymethyl-N-methylformamide) (Lide 2005). Since treatment of female silk with water eliminates male courtship response (Roberts & Uetz 2004a; Roberts unpubl.), any compounds eliciting male courtship are expected to have a high solubility in water and other polar solvents. However, this does not disqualify the identified compounds from playing a role in eliciting chemoexploratory behaviors. Roberts & Uetz (2004a) found that silk washed in methanol still elicited chemoexploratory behavior, but did not see a strong response to silk extracts. The extraction and concentration methodology used on the samples in this study may have increased the quantity of chemical(s) present in the final extract above a behavioral threshold, eliciting a chemoexploratory response. So, while we were not able to identify any components of female that elicited male courtship, we were able to successfully reestablish male searching behavior in response to female silk extracts, which has not been previously demonstrated in this species.

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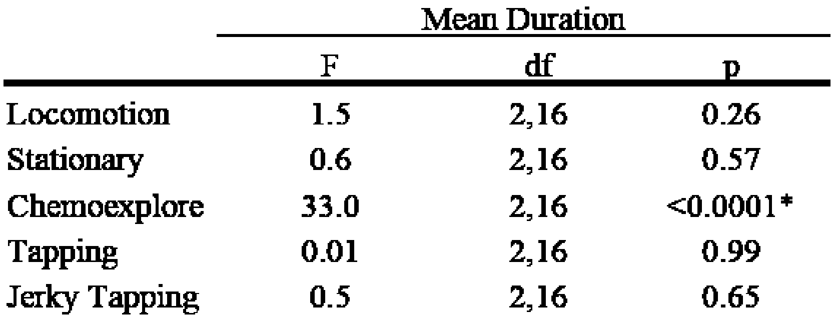


	Mean Duration		
	F	df	p
Locomotion	1.5	2,16	0.26
Stationary	0.6	2,16	0.57
Chemoexplore	33.0	2,16	<0.0001*
Tapping	0.01	2,16	0.99
Jerky Tapping	0.5	2,16	0.65

Table 4.1. Mean duration of male behaviors on silk extract treated filter paper. * indicates statistical significance following Bonferroni adjustment of the alpha level ($\alpha = 0.01$).

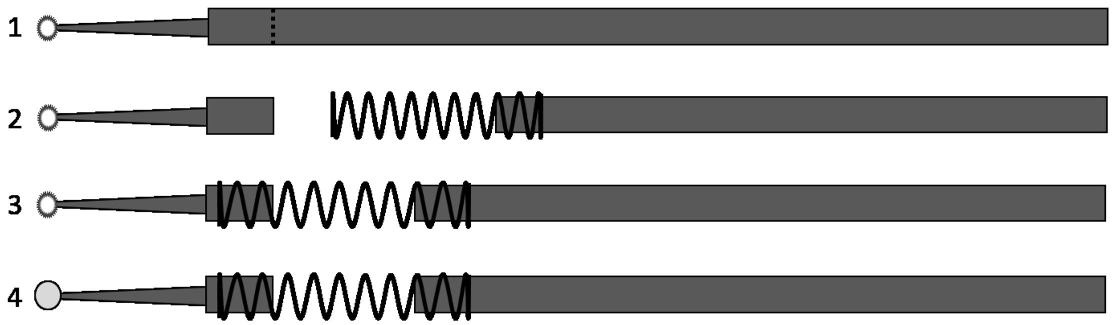


Figure 4.1. Preparation of a flexible spider wax restraint. The tip of a microbrush is removed (1) and inserted into a spring attached to a microbrush shaft (2). The tip is joined to the spring by the application of melted dental wax (3), and the bristles loaded with a droplet of wax (4) to be affixed to a spider.

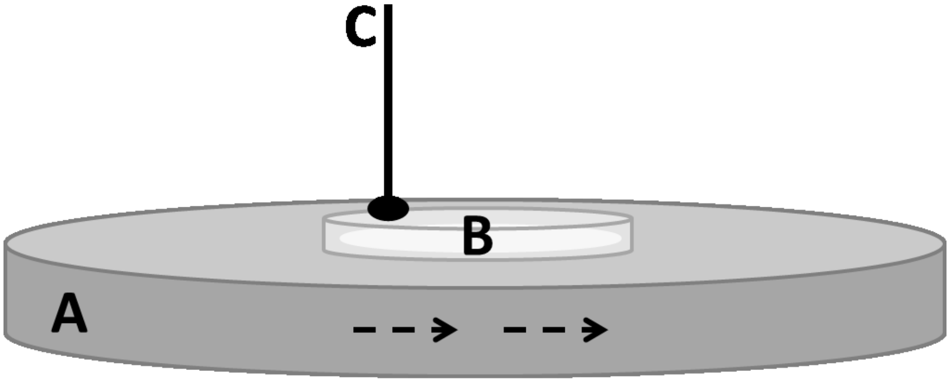
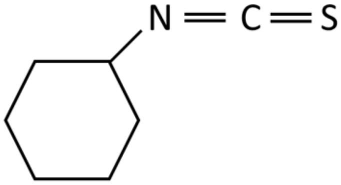


Figure 4.2. Diagram of variable speed silk collection apparatus. The turntable platform (A) rotates in the direction of the dashed arrows. A Petri dish with filter paper (B) placed in the center of the turntable was used to collect cues from a restrained spider affixed to a vertical shaft (C). The spider was oriented to allow them to walk as the substrate moved beneath them.

1



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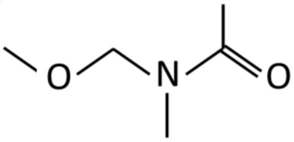

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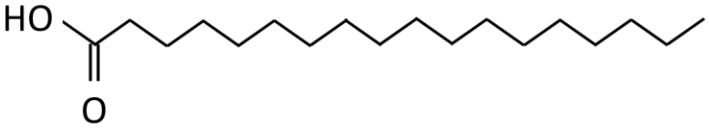

Figure 4.3. Female *Schizocosa ocreata* specific chemicals.1) cyclohexyl isothiocyanate, 2) oleonitrile, 3) N-methoxymethyl-N-methylformamide, 4) octadecanoic (stearic) acid.

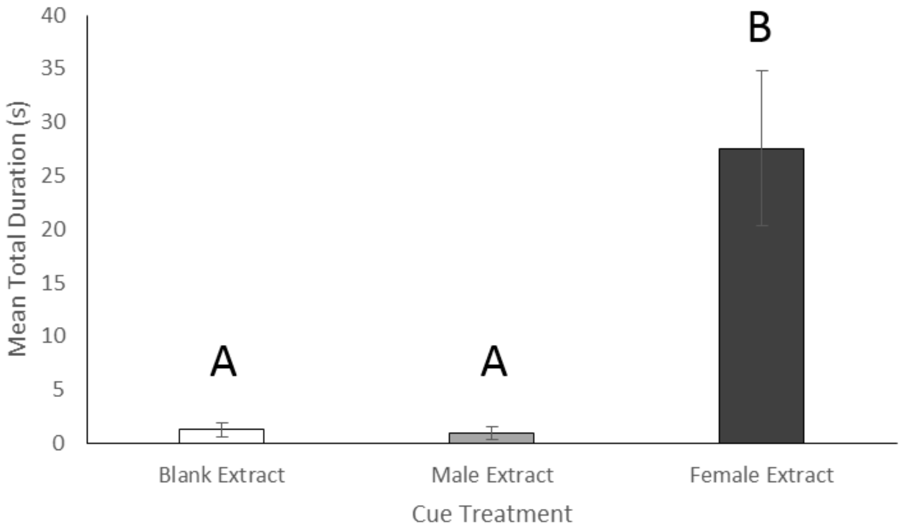

Figure 4.4. Mean chemoexploratory duration (s) (± SE) on filter paper treated with concentrated extracts of silk. Shared letters indicate no significant difference by Tukey-Kramer post hoc analysis.

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