

Male chemotactile cues are not attractive advertisements to *Pardosa milvina* (Araneae: Lycosidae) females in search of mates

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Abstract. Females use a variety of physical and behavioral features to locate potential mates and assess their quality. These male signals can be transmitted across one or multiple signaling modalities and, recently, the roles of silk-related signaling by males has received more attention. In the wolf spider, *Pardosa milvina* (Hentz, 1844), males use a visual courtship display to attract female attention and encourage sexual receptivity. We hypothesized that male chemotactile cues, including silk, feces, excretions and secretions, help females find prospective mates. We also suspected that females could use those cues to discriminate between males that differed in body condition. We monitored female reactions to isolated male chemotactile cues and to those same cues combined with visual signals provided by a sequestered male. Females did not change their activity in the presence of isolated male chemotactile cues. When males were included in the design, we verified that the courtship display was key to attracting the females and that males in good condition performed with more intensity. Surprisingly, the presence of male chemotactile cues repelled females; females approached males less often and tended to spend less time near courting males. This research verified that the visually conspicuous male courtship displays are condition-dependent and important in attracting a female. The chemotactile cues that were produced by isolated males caused females to move away from courting males, thus it is likely that any communication through chemical or tactile modalities is a more specific and individualized dialog that occurs during each courtship exchange.

Keywords: Mate choice, chemical cues, multimodal, body condition, silk

<https://doi.org/10.1636/JoA-S-19-039>

In many species, signals sent by males are the primary way in which females choose a mate. Whether they are passive ornaments (Zahavi 1975; Husak & Swallow 2011), active courtship displays (Girard et al. 2015; Zambre & Thaker 2017) or some combination thereof, these signals should tell the female something meaningful about the quality of their prospective partner. Although our tendency is to focus on conspicuous signals, environmental variation coupled with the complexity of the information that must be communicated often means that animals combine a variety of signaling modalities (Partan & Marler 1999; Candolin 2003; Hebets & Papaj 2005; Higham & Hebets 2013). For example, in the jumping spider, *Habronattus pyrrithrix* (Chamberlin, 1924), colorful males engage in a vigorous courtship display with both visual and substrate borne components (Elias et al. 2012). In the laboratory, these behaviors determine whether mating occurs; however, when males encounter females in bright sunlight, the intensity of their coloration enhances mating success (Taylor & McGraw 2013). In addition, females of the wolf spider, *Schizocosa ocreata* (Hentz, 1944), are highly sensitive to the temporal synchrony of vibratory and visual signals (Kozak & Uetz 2016) and multimodal signals supersede any preferences for unimodal signals that *S. ocreata* females may have developed early in life (Stoffer & Uetz 2017). Thus, even when the role of specific signals has been established, additional modes of communication can change the information content or convey different information depending on the circumstances.

Integrating all the information available as males engage in courtship is, of course, critical to females as they attempt to select a high-quality partner (Brooks & Griffith 2010; Schneider & Andrade 2011). However, not only do females use courtship signals to accurately assess male quality, but

they must also filter out any unreliable information that the male might include in its repertoire. This is evident in the behavior of *Pisaura mirabilis* (Clerck, 1757), a species in which males present prospective mates with a wrapped prey item as a nuptial gift. By manipulating the quality of the gift wrapping and the condition of the males, Albo et al. (2012) found that females ignored the quality of the item that was presented and consistently selected well-fed males. They argue that the gift-wrapping trait evolved to serve male interests but selection would favor females that recognize those male features that provide direct evidence of reproductive benefits (Albo et al. 2012). Examples like this reinforce the fact that detection and interpretation of the intricacies of the information provided by males are critical mechanisms by which females accomplish effective mate choice and enhance their reproductive success.

There is growing evidence that male spiders from diverse taxonomic groups deploy chemical or tactile signaling during mating, especially with the use of silk as a medium (Scott et al. 2018). While visual and vibratory information are conveyed quickly and are fairly easy to localize, a full analysis requires a clear view and/or an open substrate (Bradbury & Vehrencamp 2011; Uetz et al. 2013). On the other hand, chemical information may be more effective in a complex environment and can provide advanced information as to whether the prospective partner is acceptable or not (Gaskett 2007; Uhl & Elias 2011). Notably, female spitting spiders, *Scytodes* sp. Latreille, 1804, often kill approaching males, but odors emitted by prospective mates allow females to identify male quality early and enable less preferred males to escape cannibalism (Koh et al. 2009). Similarly, although the importance of visual and vibratory signals in the courtship of *S. ocreata* have been definitively documented (Gordon & Uetz 2011; Kozak & Uetz 2016), a recent study revealed that

females use cuticular compounds to identify the health status of prospective mates (Gilbert & Uetz 2019). Hence, it is apparent that chemical information is often important in mate assessment even when the efficacy of other, more conspicuous, features of courtship have been demonstrated definitively.

The spider *Pardosa milvina* (Hentz, 1844) (Araneae: Lycosidae) is a useful species for studying wolf spider sexual signaling with a focus on chemical communication and its interaction with the visual displays. Males use air- and substrate-borne chemical cues in order to locate females (Searcy et al. 1999; Rypstra et al. 2009), and information in those signals reveals the female's mating status (Rypstra et al. 2003) and body condition (Hoefler et al. 2009b). Upon detecting her chemically, males engage in a conspicuous display meant to attract female attention and induce receptivity for mating (Kaston 1939; Hoefler et al. 2008). Notably, the intensity of the male's activity is an indicator of his condition and those individuals that engage in more vigorous displays sire more fit offspring (Hoefler et al. 2008, 2009a). Interestingly, male and female *P. milvina* change their silk deposition patterns during courtship exchanges, which suggests that tactile and/or chemical sensory modalities might enhance communication (Havrilak et al. 2015; Khan & Persons 2015).

Our goal was to determine if virgin *P. milvina* females detect the general substrate-borne chemotactile cues (silk, feces, excretions, secretions) that males release as they roam an area and use them in mate location and evaluation. In our first experiment, we compared the activity of females on chemotactile cues isolated from males that differed in recent feeding history under the assumption that well-fed males would be more desirable (Hoefler et al. 2009a). In a second experiment, we observed female reactions to males that were held under glass and, as such, were visible but inaccessible. By manipulating the hunger level of males along with the presence or absence of chemotactile cues, we were able to explore if and how females used the visual and chemical modalities in the detection and assessment of potential mates.

METHODS

Spider collection and maintenance.—We collected spiders in corn and soybean fields at Miami University's Ecology Research Center, just north of Oxford, Ohio USA (39.532604N, 84.722998W) April through October of 2015 and 2016. Females were caught as sub-adults in the field and allowed to reach maturity in the laboratory in order to ensure their virginity, because virgin female lycosids are more sexually receptive than mated females (Fernández-Montraveta & Ortega 1990; Rypstra et al. 2003). Mature females were between 3 and 20 days post-maturation, an age at which we have observed them to readily respond to courting males. Males were caught as adults in the field and, as such, were of unknown age and mating status (as in Hoefler et al. 2009b; Rypstra et al. 2009). Male lycosid mating status does not seem to affect male courtship effort or female receptivity (Norton & Uetz 2005; Jiao et al. 2011). We housed spiders individually in circular clear plastic containers (5 cm diameter, 6 cm height) with approximately 2 cm of a damp soil and peat moss mixture covering the bottom in order to provide substrate and moisture. The spiders were maintained in an environmental

chamber with a constant temperature (25 °C), constant relative humidity (50%) and a 13 h:11 h light:dark cycle. We kept all spiders in the laboratory for at least two weeks before we used them as experimental subjects so that they could acclimate to the laboratory conditions. While they were acclimating to the laboratory, we fed the spiders two 3 mm long crickets (*Gryllobates sigillatus*) weekly. All of the crickets used as prey items were purchased from Gann's Cricket Farm (Augusta, GA, USA) and maintained communally with an *ad libitum* supply of water and commercial dog food (Ol' Roy Dog Food, Bentonville AR, USA, 21% protein).

Feeding regimes and body size.—For both experiments, we randomly assigned male spiders to one of two feeding regimes: Well-fed or Food-limited. Studies of the functional response of male *P. milvina* reveal that males rarely kill and consume more than one prey item, regardless of the number provided (Walker & Rypstra 2002). Thus, we adjusted hunger level by providing males in the Well-fed group with prey more frequently. Well-fed males were provided with two 3 mm crickets three times over the course of one week, on days 1 (the start of the feeding regime), 3, and 5. The Food-limited males were fed two crickets only on day 1 and held with access to water but no food until experiments commenced seven days later. We fed all females two crickets at the start of the experiment (day 1), and then once more the day before behavioral trials (day 7) in order to ensure they were uniform in condition. No spider was involved in more than one aspect of one experiment.

At the conclusion of the feeding regime and before experimentation began (day 7), we measured the carapace and abdomen widths of all males to the nearest 0.01 mm using an optical micrometer attached to a stereomicroscope (Wild Heerbrugg, Switzerland). These measurements were used to verify that the different feeding regimes had an effect on male body condition. The quantification of body condition has been the topic of much discussion but three approaches generally prevail (Wilgers & Hebets 2015). One approach is to calculate the ratio between a flexible measure that responds to resource acquisition and the body size, in this case the abdomen and carapace widths, and compare them in using a t-test (Anderson 1974; Jakob 1991). A second approach is to compare the measure that responds to food intake (abdomen width in our study) in an ANCOVA with treatment as the factor and with body size (carapace width) as the covariate (Garcia-Berthou 2001). The third common approach is to regress the flexible measure (abdomen width) on body size (carapace width) and compare the residuals generated for the animals in each treatment group in an ANOVA (Jakob et al. 1996). We also had measurements of 50 males collected from the same areas at the Ecology Research Center between April and October of 2014. We compared the ratio of their abdomen to carapace widths to our treatments using an ANOVA followed by Tukey's HSD post-hoc test to verify that our treatments were reflective of the range of feeding levels that the animals experienced in the field. We deployed all three approaches using the R software (R Core Team 2016).

Isolated chemotactile cue choice trials.—In the first experiment, we deployed a design used to quantify activity in response to different substrates in past studies with *P. milvina* (Persons & Rypstra 2001; Persons et al. 2001, Evans et al.

2010; Hoffman et al. 2016). The substrates we used contained chemotactile cues, which consisted of silk and feces along with any other excretions and secretions produced by the males as they occupied the area (Persons et al. 2001). We collected cues on filter paper (Grade 1, Whatman). The bottom of cylindrical containers, 15 cm in diameter, were lined with filter paper discs that had a small hole in the center (4 cm in diameter). Into that hole, we placed a damp cotton ball on top of a plastic cap so that the spiders would have access to water during cue deposition. We released males into the containers, covered them, and placed them into an environmental chamber. The males were allowed to roam freely over the filter paper for 24 hr during which time they deposited their chemotactile cues. The males were removed and returned to maintenance conditions. The filter paper lining was used to present chemotactile cues to the females. We produced filter paper with cues from Well-fed and Food-limited males and we also used Blank pieces that had never been in contact with a spider.

We prepared arenas by cutting each piece of filter paper in half and placing different halves side-by-side in a circular trial arena (15 cm diameter) (see diagrams in Persons & Rypstra 2000, 2001; Persons et al. 2001). Papers were cut with a razor cutter to minimize disturbance of the silk. We established three pairings: cues from Well-fed males paired with cues from Food-limited males ($n = 19$), cues from Well-fed males paired with Blank filter paper ($n = 19$), and cues from Food-limited males paired with Blank filter paper ($n = 19$). The side that each cue was placed on was randomized with each trial to control for any side bias. We then situated the arena under a video camera (Panasonic PV-DV73, Japan) located in an isolated chamber.

To initiate a trial, we placed a female at the center of the arena inside an inverted plastic vial (4 cm diameter) and allowed her to acclimate for 3 minutes. We then removed the vial to release the female and video recorded her movements for 15 minutes. At the end of the trial, the female was removed and placed back into her container, the used filter paper halves were discarded, and the arena was washed with 70% ethanol to remove any traces of chemical cues.

We quantified female movement using the EthoVision software (EthoVision[®] XT, Noldus, The Netherlands), which tracked the movement of the females in each region of the arena at a rate of 10 frames per second. For each filter paper half, we quantified four activity metrics: (1) the total time spent on each side (s), (2) the proportion of time spent moving when on each side, (3) the distance females moved (cm) when on each side, and (4) average velocity (cm/s) when on each side. Any trials in which the females did not come into contact with both halves of the arena were excluded from the analyses.

Females varied considerably in their activity patterns, which meant that the data were not normally distributed. As a result, we applied non-parametric statistics. We examined the female's reactions to male chemotactile cues using a two-pronged approach. First, we compared each of the four female activity metrics (duration, distance, proportion of time moving, average velocity) for each of our three paired treatments (Well-fed vs Food-limited, Well-fed vs Blank, Food-limited vs. Blank) using Wilcoxon matched pairs tests. Next we verified that the activity of the females on a particular stimulus was not impacted by the cues on the other side of the

arena in a Mann-Whitney U-tests (i.e., Well-fed compared with Well-fed, Food-limited compared to Food-limited, Blank compared to Blank) (all $P > 0.3$). We then combined all the female activity data on Well-fed cues, Food-limited cues, and Blank paper (regardless of the treatment that accompanied them) and performed a Kruskal-Wallis test. We note that running tests on multiple activity metrics increases the likelihood of a Type 1 error. For both approaches, we report the actual p-value generated by the statistical test and, r , an effect size measurement adjusted for use with non-parametric data (Fritz et al. 2012; Lenhard & Lenhard 2016). A value of r less than 0.1 corresponds to a non-existent effect, when r is between 0.1 and 0.3 any effect is considered small, when r is between 0.3 and 0.5 the effect is identified as moderate, and any value of r larger than 0.5 translates to a large effect (Fritz et al. 2012; Tomczak & Tomczak 2014). Statistical analyses were conducted using the R software (R Core Team 2016).

Multimodal cue trials.—For the second experiment, we investigated whether chemotactile signals from males that differed in condition altered the female's response to male courtship displays. To do this, we released females in arenas with sequestered males that were either surrounded by their chemotactile cues or no cues and observed them. The condition of these males was manipulated using the same feeding regimes as in the first experiment. Thus, we included four treatments: Well-fed males surrounded by their cues ($n = 20$), Food-limited males surrounded by their cues ($n = 21$), Well-fed males surrounded by Blank filter paper ($n = 19$), and Food-limited males surrounded by Blank filter paper ($n = 22$). Cue males were placed in cylindrical containers (9 cm diameter) lined with filter paper (Grade 1, Whatman) to collect the silk, feces, excretions and secretions prior to the trial. A small amount of wet cotton on a plastic cap was included so that the spiders had access to water. The containers were covered and placed in the environmental chamber for 24 hours. The Blank males were kept in their containers until the trials began.

Trials were conducted in a manner that mimicked a design used to explore male reactions to females (Rypstra et al. 2009; Fig. 1). Rectangular containers (31 cm x 16.5 cm x 7.8 cm) were lined with construction paper to provide an even and uniform surface across all containers (Fig. 2). We sequestered males under inverted clear glass vials (1.75 cm diameter) on top of the circle of filter paper containing their cues (Cue males), or on paper that had not been in contact with any spider (Blank males) (Fig. 1). The males were located 8 cm from one end of the arena and females were introduced 7 cm from the opposite end. Females were held underneath inverted opaque plastic vials (2.2 cm in diameter) (Fig. 1), allowed to acclimate for 3 minutes, and then released. Over the next 2 hours, we recorded how long it took the female to make contact with the filter paper surrounding the male (latency), the amount of time the female spent in contact with the filter paper surrounding the male (time on), the number of times the female contacted the male's vial (approaches), and the number of times the male completed a set of courtship displays (courtship bouts). We note that male courtship consists of distinct leg raises and body shakes (Rypstra et al. 2003) but, in this experimental situation, we were not able to count the frequency of these specific behaviors. Nevertheless, we were

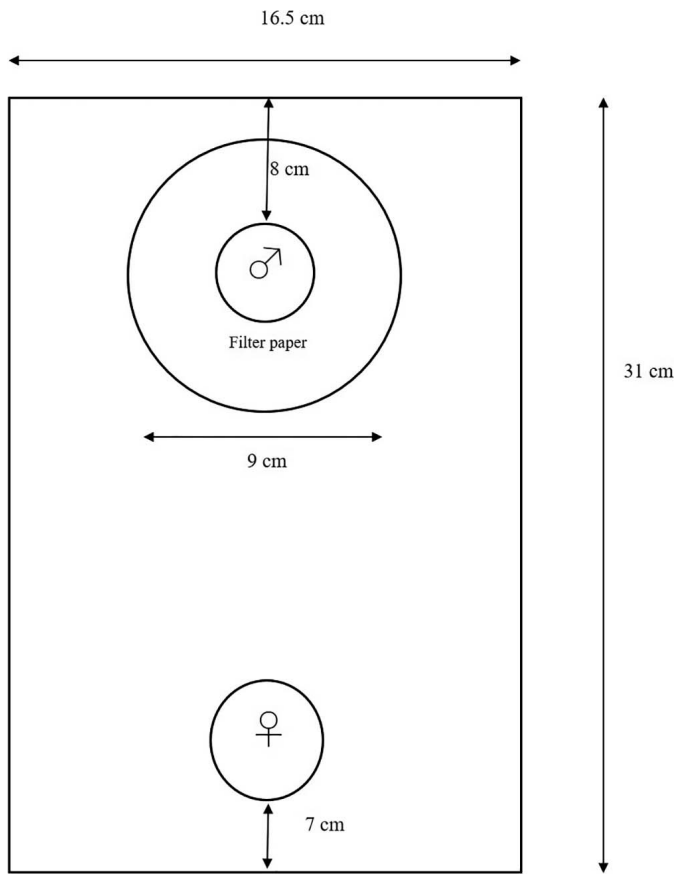


Figure 1.—Experimental arena design for the multimodal cue experiment. The ♂ indicates where a Well-fed or Food-limited male was contained under a clear glass vial surrounded by filter paper which either contains his cues or was Blank. The ♀ indicates where the female was released after being acclimated under an opaque vial.

able to determine when he was actively courting and when he was not. When periods of courtship activity were separated by more than 10 seconds, we considered them distinct courtship bouts.

The data recorded for the latency of females to contact the filter paper and the time they spent on the filter paper surrounding males were not normally distributed. Hence, we used the Kruskal-Wallis test to compare the four treatments (including Well-fed and Food-limited males, each with and without cues). We then combined all the treatments with cues present and compared them to all of the blank trials using a Wilcoxon rank-sum test. In each of these instances, we also calculated r , an effect size adjusted for use with non-parametric data. These analyses were conducted with R software (R Core Team 2016).

The number of times the female approached the male and the number of courtship bouts both matched the Poisson distribution. Hence, we were able to use a generalized regression with the Poisson distribution to determine if male feeding status or the presence or absence of his chemotactile cues affected the frequency of these activities. We initially ran the analyses with interactions included and removed them if they were not significant. To better understand how the male mating display itself was affecting females, we used Spear-

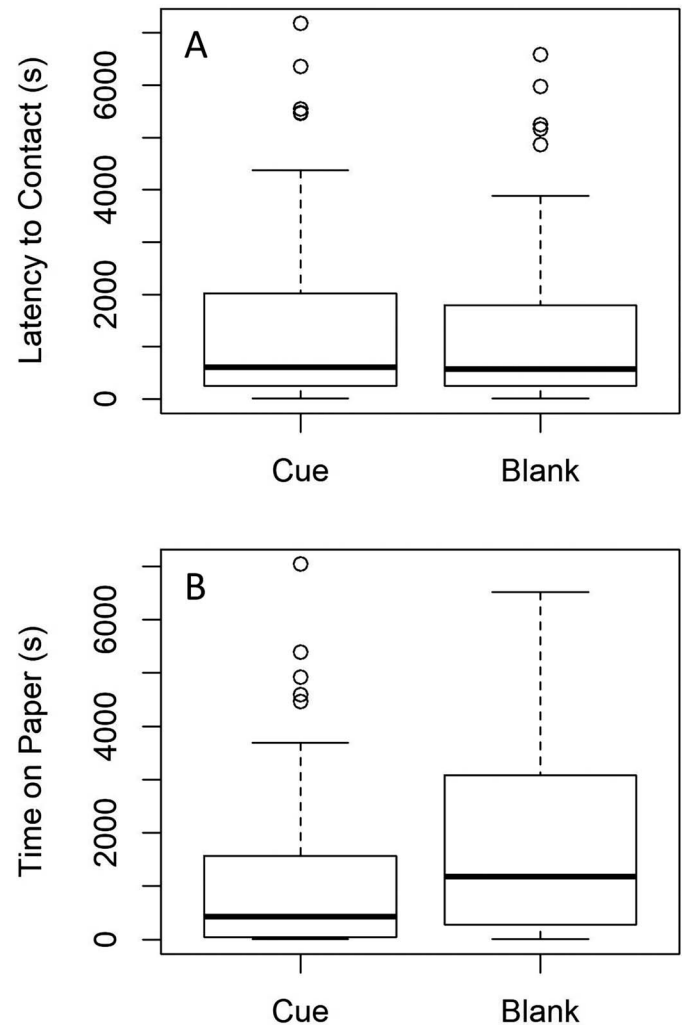


Figure 2.—Times (s) for female *P. milvina* reactions to males that were either surrounded by filter paper containing male chemotactile cues (Well-fed and Food-limited combined) or those that were surrounded by Blank filter paper. (A) Latency to contact = the time (s) that it took females to find the filter paper circle and (B) Time on paper = the total time females spent on the paper circle surrounding the male. The black line indicates the median, the box edges are at the 25th and 75th percentiles, and the whiskers extend to the highest or lowest values that are within 1.5 times the interquartile range. Circles indicate the location of data outside of this range.

man's ρ to assess correlations between the number of male courtship bouts and female latency to approach the male, time on the filter paper, and the number of times she approached the male using the R software (R Core Team 2016).

RESULTS

Body condition.—Across all experiments, males maintained on the Well-fed diet ($n = 77$) had a greater final body condition ratio (abdomen/carapace) than males on the Food-limited diet ($n = 81$) (Well-fed abdomen/carapace width: 1.96 ± 0.03 ; Food-limited abdomen/carapace width: 1.72 ± 0.02 , $t = 6.48$, $P < 0.0001$). This difference was verified by comparing abdomen width between treatments in an ANCOVA with

Table 1.—Activity metrics (mean \pm SE) for *Pardosa milvina* females in containers with different pairs of male cues: Well-fed with Food-limited, Well-fed with Blank, Food-limited with Blank. Duration is the time spent on each cue, Distance is the centimeters moved on each cue, Proportion moving is the proportion of time spent moving out of all the time spent on each cue, and Velocity is the average speed of the female when on each cue. Results of Wilcoxon matched pairs tests (V , P) and effect sizes (r) are also provided.

Male cue pair	n	Duration (s)	Distance (cm)	Proportion moving	Velocity (cm/s)
Well-fed vs. Food-limited	16	492.21 \pm 51.67	241.12 \pm 28.49	0.12 \pm 0.02	0.57 \pm 0.08
Wilcoxon		399.74 \pm 52.05 $V=84$, $P=0.433$ $r=0.146$	242.50 \pm 46.57 $V=71$, $P=0.900$ $r=0.027$	0.15 \pm 0.03 $V=52$, $P=0.433$ $r=0.146$	0.67 \pm 0.09 $V=48$, $P=0.323$ $r=0.183$
Well-fed vs. Blank	17	434.80 \pm 43.54	267.26 \pm 36.03	0.13 \pm 0.02	0.65 \pm 0.07
Wilcoxon		446.23 \pm 46.83 $V=67$, $P=0.678$ $r=0.077$	280.82 \pm 38.51 $V=64$, $P=0.579$ $r=0.101$	0.15 \pm 0.03 $V=80$, $P=0.890$ $r=0.028$	0.75 \pm 0.11 $V=74$, $P=0.927$ $r=0.020$
Food limited vs. Blank	17	405.67 \pm 40.50	322.22 \pm 43.97	0.21 \pm 0.04	0.90 \pm 0.12
Wilcoxon		460.71 \pm 43.44 $V=51$, $P=0.244$ $r=0.207$	347.23 \pm 38.16 $V=59$, $P=0.431$ $r=0.142$	0.18 \pm 0.03 $V=106$, $P=0.174$ $r=0.239$	0.86 \pm 0.10 $V=107$, $P=0.159$ $r=0.248$

carapace width as a covariate ($F = 20.469$, $P < 0.0001$) and in a t-test of the residuals generated from a linear regression of abdomen width on carapace width ($t = 5.57$, $P < 0.0001$). The abdomen/carapace ratio of field-caught males from the summer of 2014 were intermediate between our two treatments (1.86 ± 0.05). When we compared the ratios in an ANOVA, the overall model was significant ($F = 12.16$, $P < 0.0001$) but the values for field caught animals were not significantly different from males in either our Well-fed or Food-limited treatments (Tukey HSD post-hoc test, $P > 0.05$).

Isolated chemotactile cue choice trials.—We documented the activity of 57 females (19 on each of the three treatment pairs) but excluded seven of them because the females did not contact both stimuli. We excluded three from the treatment where Well-fed cues were paired with Food-limited cues, two from the pairing of Food-limited cues with Blank filter paper and two from the pairing of Well-fed cues with Blank filter paper. Overall, females did not alter their behavior when in the presence of male chemotactile cues, regardless of the condition of the male that produced them (Tables 1, 2). The variables we quantified included the time spent on each side of the arena, the proportion of time spent moving on each side, the distance travelled on each, and the average velocity of the female on each side. None of these activity metrics were significantly different for any pair of cues (all comparisons $P > 0.1$) (Table 1). The only hint that females altered their behavior in the presence of cues was in the treatment with cues from Food-limited males paired with Blank filter paper; the effect sizes for

all of the activity metrics we quantified were the highest we observed in this experiment but still had to be categorized as small ($0.14 < r < 0.25$) (Table 1). When we pooled the activity data for females on Well-fed cues, Food-limited cues, or on Blank filter paper, regardless of the treatment that was on the other side of the arena, there were no statistically significant differences (all $P > 0.18$) and the effect sizes for all comparisons were small ($0.12 < r < 0.21$) (Table 2). Overall, it seems that any effects of isolated male chemotactile cues on female activity were miniscule.

Multimodal cue trials.—In our second experiment that included sequestered males in the arena, we uncovered no differences that could be attributed to the presence of cues or the feeding status of the males in the time it took the females to approach the males ($\chi^2 = 0.51$, $P = 0.92$, $r = 0.027$) (Table 3) or the time then spent on the filter paper near the male ($\chi^2 = 4.70$, $P = 0.20$, $r = 0.198$) (Table 3). When we collapsed the Well-fed and Food-limited treatments and compared trials with and without cues present, the presence of male cues did not affect the time it took females to come into contact with the filter paper surrounding him ($W = 787$, $P = 0.80$, $r = 0.027$) (Fig. 2A) but there was a hint that the presence of male chemotactile cues reduced the time she spent near the males (Wilcoxon rank-sum test, $W = 567$, $P = 0.05$, $r = 0.354$) (Fig. 2B).

Females approached males significantly more often when the arena was clear of chemotactile cues (Poisson regression $\chi^2 = 11.44$, $P = 0.0007$, $r = 0.374$) (Table 3) but they did not respond to the feeding status of the male (Poisson regression

Table 2.—Activity metrics (mean \pm SE) for *Pardosa milvina* females on the three substrates with all data for each cue type combined across treatments. Substrates include cues from Well-fed males, from Food-limited males or Blank paper with no cues. Duration is the time spent on each cue, Distance is the centimeters moved on each cue, Proportion moving is the proportion of time spent moving out of all the time spent on each cue, and Velocity is the average speed of the female when on each cue. The χ^2 and P derived from Kruskal-Wallis tests as well as the effect size (r) are included.

Activity metric	Well-fed	Food-limited	Blank	χ^2	P	r
Duration (s)	454.22 \pm 33.48	418.24 \pm 32.22	437.99 \pm 31.48	1.339	0.512	0.128
Distance (cm)	244.91 \pm 22.89	265.17 \pm 32.26	296.51 \pm 26.64	1.820	0.403	0.149
Proportion moving	0.125 \pm 0.016	0.180 \pm 0.025	0.164 \pm 0.019	3.453	0.178	0.205
Velocity (cm/s)	0.570 \pm 0.054	0.710 \pm 0.078	0.750 \pm 0.073	3.196	0.202	0.197

Table 3.—The time it took females to approach males (latency), the time females spent near the males (time on), the number of times females approached males, and the number of courtship bouts males performed (mean \pm S.E.) in multimodal trials. Data are categorized by the feeding status of the male and the presence or absence of chemotactile cues.

Treatment	Latency (s)	Time on (s)	Approaches	Courtship bouts
No cues present				
Well-fed	1452 \pm 434	1951 \pm 469	2.6 \pm 0.6	3.7 \pm 1.4
Food-limited	1281 \pm 392	1629 \pm 398	3.8 \pm 1.0	2.1 \pm 0.6
Cues present				
Well-fed	1307 \pm 426	1421 \pm 396	2.9 \pm 0.7	2.3 \pm 0.7
Food-limited	1626 \pm 468	1113 \pm 415	1.2 \pm 0.5	1.0 \pm 0.3

$\chi^2 = 0.28$, $P = 0.60$, $r = 0.058$) (Table 3). Our data suggest that males and females communicated with one another as the number of courtship bouts was negatively correlated with the female's latency to approach (Spearman's $\rho = -0.36$, $P = 0.0001$) (Fig. 3A), and positively correlated with the number of approaches (Spearman's $\rho = 0.67$, $P < 0.0001$) (Fig. 3B), as well as the time she spent on the filter paper surrounding him (Spearman's $\rho = 0.55$, $P < 0.0001$) (Fig. 3C). Consistent with these results, the number of courtship bouts was lower when male cues were present (Poisson regression $\chi^2 = 15.23$, $P < 0.0001$, $r = 0.431$) (Table 3) and there was an effect of male feeding status on the number of courtship bouts that occurred (Poisson regression $\chi^2 = 19.21$, $P < 0.0001$, $r = 0.484$). Well-fed males engaged in more courtship than Food-limited males (Table 3).

DISCUSSION

Our results suggest that female *Pardosa milvina* are unlikely to use male chemotactile cues to find prospective mates in a complex field environment but our data reveal some insights into the multimodal exchange between the sexes during courtship and mate selection. There were no substantial shifts

in female activity when in the presence of chemotactile cues from isolated males (Tables 1, 2). Moreover, when males were visible in the arena, the chemotactile cues seemed to reduce female interest; females approached courting males less frequently (Table 3) and spent less time near sequestered males when cues were present (Fig. 2B). Because we primarily wanted to determine the role that chemotactile cues played in helping females find males, the cues we manipulated were produced when males were isolated with no direct information that females were in the area. While our experiments suggest that these cues do not serve as male advertisements, we observed a mutual exchange between male courtship and female attraction (Fig. 3) that was consistent with the prevailing understanding of mate assessment in this species (Rypstra et al. 2003, 2009, 2016). Likewise, we documented a reduction courtship related to hunger (Table 3), which has also been shown to have fitness implications for *P. milvina* (Hoeffler et al. 2008, 2009a).

We were surprised that we did not see larger differences in female activity when they were placed on male chemotactile cues in our first experiment. We have no doubt that females detected the male cues because female *P. milvina* are very sensitive to similar cues from both con- and hetero-specific spiders (Persons et al. 2001; Lehmann et al. 2004; Hoffman et al. 2016). This sensitivity drew us to the idea that virgin females might exploit male cues as signals while searching for prospective partners but, if that were the case, we would have expected females to shift some aspect of their activity upon encountering those stimuli. However, we uncovered no significant impact of cues on any of the activity metrics we documented and the effect sizes were low enough to make it unlikely that this signaling mode plays an important role in mate location in the field. The persistence of these kinds of chemotactile cues in wolf spiders is fairly short in the face of environmental factors such as moisture and heat (Wilder et al. 2005) and the reactions of females in a controlled laboratory situation would need to be strong and immediate if we were to reliably extrapolate them to a natural situation.

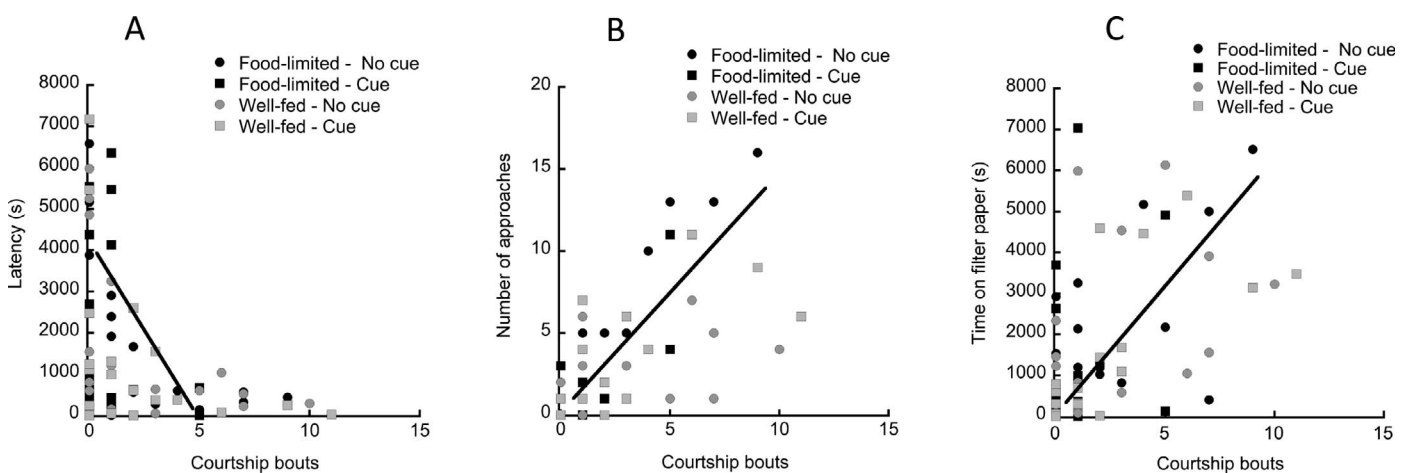


Figure 3.—The number of courtship bouts that males performed and (A) the latency for the female to approach him, (B) the number of times females approached males and (C) the time females spent on the filter paper disc surrounding males. Data are categorized by the feeding regime of the male and the presence or absence of chemotactile cues on the filter paper disc. The lines represent the trends to illustrate the direction of the correlations and are not regression lines.

It was even more surprising that, when courting males were sequestered in the arena, the presence of chemotactile cues repelled females. The males in our experiment were field-collected and of unknown age or mating status and, as such, it may be that the added chemical cues enabled these virgin females to determine that they were unacceptable and move away, whereas in the absence of the chemotactile cues, females needed to conduct a more critical assessment based on his behavior. We used field-caught males because past studies have not reported any bias by virgin females against mated males and have documented mating acceptance rates of up to 97% in no choice tests (Rypstra et al. 2003). However, we are unaware of studies explicitly testing for differences in the relative desirability of field-caught adult, potentially mated, males to virgin female *P. milvina*.

It is important to note that *P. milvina* males and females engage in a subtle silk dialog before and during courtship (Havrilak et al. 2015; Khan & Persons 2015). Specifically, courting males produce different types of silk than non-courting males (Khan & Persons 2015) and this “courtship” silk causes females to alter the amount and nature of silk that they deposit (Khan & Persons 2015). Additionally, there is evidence that *P. milvina* females directly signal prospective mates as females deposit significantly more dragline silk on substrates when they see, but cannot reach, males that are not courting or courting at a very low rate (Havrilak et al. 2015). Assuming this targeted give and take is the norm, the females in our study were likely confused by the mismatch between the general dragline silk in the chemical cues we manipulated and the fact that there was no buildup of “courtship silk”. As the interaction played out, failure to receive silken confirmation of the male’s species and/or receptivity may be what caused females to be more cautious and retreat from our sequestered males. By the same token, in our treatments with no added chemotactile cues, there was no conflicting or confirmatory information available to the females. In these cases, females approached sequestered males more often and stayed closer longer, presumably presenting the “attraction” silk, as was observed in the experiments conducted by Havrilak et al. (2015). Thus, interpretation of our results in the context of these other studies leads to the conclusion that, once males and females have seen and identified one another, a targeted dialog ensues, conveyed in the silk deposition pattern. While males and females deploy multiple sensory modalities in their efforts to assess one another’s quality and receptiveness, it is a much more intimate exchange than we expected.

Our attention was directed toward visual and chemical signaling, but it is likely that there is a vibratory component to male signaling in *P. milvina* as well. This species detects and discriminates among predators based on vibratory information (Sitvarin et al. 2016), and the vigor with which males engage in leg raises and body shakes during courtship probably produces detectable vibrations in the substratum. However, we expect that these vibratory signals would be synchronous with the visual display and, as such, uncoupled from the chemotactile influences we set out to explore. If this is true, then consideration of this additional sensory modality would not affect the interpretation of the data presented here. On the other hand, the vial that sequestered the male could have dampened transmission of vibratory signals through the

substratum and the silk and cues on the filter paper may have altered the nature of any vibratory information in a manner that affected the ability of females to accurately interpret the situation. In this way, a subtle interaction among the visual, vibratory and chemical aspects of the courtship signal may have been missing and that missing link could have been what drove females away from the males. However, this species thrives in structurally complex environments where signals in different modalities would often be asynchronous or totally uncoupled. This knowledge led us to focus primarily on male chemotactile cues and their potential role in attracting females. Our experiments suggest that male cues play a minor role in the initial attraction and assessment efforts by females. Further investigations should explore if and how these chemotactile cues might amplify or modulate the information conveyed through other sensory channels.

In the isolated cue experiments, we manipulated the filter paper after the cues were deposited, which may have disturbed some of the features of silk deposition or chemical properties. However, the half circles were 88 cm² in total area and most of the cued area was untouched. We expect that, in an area of this size, enough of the signal would be intact to cause some activity change if it were an important aspect of the female’s search for a partner. After all, in a natural situation, these same cues would not be deposited on a clear clean surface, but on soil, along with living and dead plant material, and they would be interrupted by other signals and environmental perturbations. As result, some disturbance in an area of this size would likely be quite common. On the other hand, *Schizocosa rovneri* Uetz & Dondale, 1979 males adjust their signaling in response to female feedback and those that were able to best match their signal with the substrate were more successful (Sullivan-Beckers & Hebets 2014). It would be interesting to determine if *P. milvina* females prioritize different signaling modalities or interpret them differently depending on the substrate and the way that the male responds.

Past studies have established that *Pardosa milvina* males search for females and, upon detecting them through air- and substrate-borne cues, commence visually conspicuous courtship displays that drive mate selection (Searcy et al. 1999; Rypstra et al. 2003, 2009, 2016). Here, we document the importance of the visual exchange between the sexes. Our males were held under glass vials which would greatly restrict the entry of air-borne pheromones and eliminate access to female chemotactile cues, yet all of these males engaged in active courtship that was modulated to the females’ behavior. Likewise, females moved toward males more quickly, approached more often, and stayed near the males that courted more. These results confirm that there is a visual exchange of information uncoupled from the silk or chemical dialog we speculated about earlier in this discussion. We suspect that the lower levels of courtship observed when males were on their own cues was due to the reduced interest of the females and not some direct influence of, or association with, their own mixture of silk and chemicals. It is much more likely that the visual information of an approaching female, in good condition and approaching him repeatedly, encouraged his activity.

Past studies have demonstrated that male condition influences courtship rate and that males confronted with high quality females tend to court at their maximum intensity (Hoefler et al. 2009a). Here we saw that Well-fed males engaged in more courtship bouts than their hungry counterparts and that over all treatments, females responded to the frequency of those spells of courtship activity (Fig. 3). Presumably, if we allowed these interactions to be totally unfettered, we would have seen a stronger connection between male condition or quality and female responses, as has been observed in past studies (Hoefler et al. 2009a; Rypstra et al. 2016).

In conclusion, it seems that the chemotactile cues left behind by *P. milvina* males as they roam an area do not function to attract females. It is possible that these cues allowed females to assess and perhaps reject males quickly when the males can be seen. However, these results coupled with other studies (Havrlik et al. 2015; Khan & Persons 2015) suggest that silken signaling from males is primarily important only after male courtship has begun. Nevertheless, even with these non-courting cues present and the male held under a glass vial, we were able to document a give and take between males and females. This exchange included enough stimuli from the female to elicit courtship and enough courtship from the male to encourage her attention. Future investigations need to explore the specific conditions under which silk and other chemical and vibratory signals are produced and propagated in order to further our understanding of the dynamics of communication during mating in wolf spiders.

ACKNOWLEDGMENTS

We appreciate the help we received from Alexander Berry, Lacey Campbell, Lucas Erickson, Jake Godfrey, and Gabrielle Stanley in collecting spiders, executing experiments, reviewing various drafts of this paper and for providing moral support. Drs. N.G. Solomon and B. Keane provided valuable input throughout the project as well. This project was executed as a part of the requirements of Master of Science degree at Miami University, Oxford, OH USA. Funding was provided by Miami University's Department of Biology, and Hamilton Campus.

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Manuscript received 12 July 2019, revised 28 May 2020.