Discrimination of airborne pheromones by mate-searching male western black widow spiders (*Latrodectus hesperus*): species- and population-specific responses

Michael M. Kasumovic and Maydianne C.B. Andrade

Abstract: Males of many web-building spiders abandon their webs at maturity to search for a potential mate. Since wandering can be very risky, and females are often widely distributed, males should use any cues that might ensure rapid and accurate location of conspecific females. Although it has long been assumed that mate-searching male spiders locate females using species-specific airborne pheromones released from webs, few studies have experimentally examined this phenomenon in the field. Our results show that male western black widow spiders (*Latrodectus hesperus* Chamberlin and Ivie, 1935) are attracted to females’ webs by an airborne cue released from the web, can distinguish between conspecific and heterospecific females, and can discriminate between webs produced by conspecific females from different geographical populations. The latter result demonstrates a partial premating block to fertilization between populations at the edges of the species range. Complementary interpopulation laboratory matings suggest that there may also be a postmating block to fertilization, as these copulations did not result in viable offspring. This study provides experimental field evidence of male attraction by airborne pheromones released from females’ webs, shows the potential importance of these pheromones in species discrimination in black widow spiders, and suggests that northern and southern populations of *L. hesperus* may be incipient biological species.

Résumé : Les mâles de plusieurs araignées constructrices de toiles abandonnent celles-ci à maturité pour aller à la recherche d’un partenaire potentiel. Parce que l’errance peut poser de nombreux risques et que les femelles sont souvent répandues sur de grandes surfaces, les mâles ont avantage à utiliser tout signal qui peut leur permettre de localiser avec rapidité et précision les femelles de leur espèce. Bien qu’on croit depuis longtemps que les mâles en quête de partenaire trouvent les femelles au moyen de phéromones spécifiques à l’espèce émises par les toiles, ce phénomène n’a que rarement été étudié expérimentalement en nature. Nous démontrons que les mâles du latrodecte *Latrodectus hesperus* Chamberlin et Ivie, 1935 de l’ouest sont attirés vers les toiles des femelles par un signal transmis dans l’air provenant de la toile, qu’ils peuvent distinguer entre les femelles conspécifiques et les femelles des autres espèces et qu’ils peuvent reconnaître les toiles de femelles conspécifiques appartenant à des populations géographiques différentes. Cette dernière observation indique l’existence d’une barrière partielle avant l’accouplement entre les populations aux limites de l’aire de répartition de l’espèce. Des accouplements supplémentaires entre les populations en laboratoire indiquent qu’il y a probablement aussi une barrière à la fécondation après l’accouplement, car ces accouplements n’ont produit aucun rejeton viable. Notre étude apporte des preuves de terrain que les mâles sont attirés par les signaux transmis dans l’air provenant des toiles des femelles; elle montre aussi l’importance potentielle de ces phéromones dans la reconnaissance de l’espèce chez les latrodectes et laisse croire que les populations boréales et australes de *L. hesperus* sont des espèces biologiques en formation.

Introduction

Accurate species recognition is an important component of mate identification, as it prevents hybridization with genetically incompatible heterospecifics. Recognition involves the use of species-specific cues, which may be visual, acoustic, tactile, or olfactory in different groups, during mate attraction or courtship. Species identification in spiders is particularly interesting because they utilize several different modalities to convey this information, such as complex visual displays, acoustic displays, and (or) vibrational signalling (for a review see Krafft 1982). Spiders also use chemical signals, which is presumed to be one of the least evolutionarily derived forms of communication, in mating. The chemical composition of pheromones bound to female webs has recently been identified (Schulz and Toft 1993;
Papke et al. 2000), and behavioural studies have revealed the importance of these contact pheromones in the recognition of conspecifics and the initiation of male courtship behaviours (Trabalon et al. 1997; Papke et al. 2001; Tichy et al. 2001). Contact pheromones can also provide male spiders with information about the sex, sexual maturity, and the mating status of females (Riechert and Singer 1995; Searcy et al. 1999; Papke et al. 2001). In some of these groups, however, contact pheromones trigger courtship behaviour in heterospecific as well as conspecific males, suggesting that these pheromones alone are not likely to be useful in preventing costly mating mistakes (e.g., Ross and Smith 1979). These studies may omit a necessary step in species recognition (i.e., discrimination by mate-searching males prior to web contact).

Contact pheromones require close proximity between individuals before identification can occur, and thus, cannot be used to attract males over long distances. This may be problematic for male web-building spiders because they must often travel through dangerous terrain to reach the web of a potential mate (e.g., Henschel 2002) and experience high mortality during this time (e.g., Vollrath and Parker 1992; Andrade 2003). The risk of mortality during mate-searching should impose strong selection on males to use any available cues that might facilitate rapid and accurate identification of potential mates at a distance. Selection may also favour the production of airborne pheromones by sedentary females, particularly if webs are widely distributed and encounter rates with males are low. Thus, airborne pheromones may be an important, but little-studied, aspect of precopulatory communication for arachnids (Pollard et al. 1987).

A few studies have examined the importance of airborne pheromones in spiders; these have demonstrated that airborne chemicals released from females’ bodies attract males and can trigger courtship (Tietjen and Rovner 1982; Searcy et al. 1999; Papke et al. 2001). Although this may be sufficient to attract males, in web-building spiders the web itself (with its relatively large surface area) could provide a more efficient means for releasing attractive airborne chemicals. Studies of black widow spiders (*Latrodectus* spp.) suggest that females incorporate pheromones into their webs to facilitate web location by males (Ross and Smith 1979; Anava and Lubin 1993), but these studies included direct male contact with web-bound chemicals. To date, the majority of studies examining the efficacy of chemical signals in spiders have experimentally placed males directly on webs, examined the influence of chemicals released by the female’s body, or allowed direct contact between males and females. Thus, there is little experimental evidence that airborne pheromones released from webs attract males.

In this field study, we demonstrate that western black widow males (*Latrodectus hesperus* Chamberlin and Ivie, 1935) are attracted to females’ webs by airborne pheromones and that they discriminate conspecific female webs from those of heterospecific females (*Latrodectus hasselti* Thourel, 1870) using these airborne cues. We placed empty webs constructed by virgin female *L. hesperus* and *L. hasselti* at a location where *L. hesperus* spiders were common and monitored the arrival of mate-searching males at these webs. We included webs built by females from two geographically separated populations of *L. hesperus* (British Columbia, Canada, and Arizona, USA), and our results showed that males not only discriminate conspecific females from heterospecific females but were also disproportionately attracted to webs built by females from their own population. This partial premating block to fertilization suggests that these populations may be incipient species; a hypothesis supported by the complementary laboratory pairings, which shows that matings across these populations are less likely to result in viable offspring than are matings within a population.

### Materials and methods

#### Study site and study species

This study was completed between May and June 2003, just outside Lac Du Bois Provincial Park in Kamloops, British Columbia (50°43’40.47″N, 120°24’15.27″W). Female *L. hesperus* commonly inhabit desert habitat and, at our field site, were most often found on the side of rocky outcrops and hillside small cavities in the rocks or soil. Females build refugia within these gaps and cob webs on the rock face outside of their refugia for prey capture. Our study site consisted of two large rock outcrops, one approximately 50 m below the first. The first outcrop was 145 m long, covered an area of 860 m², and contained 74 female’s webs, while the second outcrop was 170 m long, covered an area of 1030 m², and contained 139 female’s webs (Figs. 1A, 1B). Webs were initially identified by their distinct structure (e.g., Szlep 1965), which we later confirmed as webs from *L. hesperus* because all contained females and (or) egg sacs. Webs were mapped by measuring the distance from each web to two points of known location using a laser distance meter (Leica Disto Lite; Leica Geosystems, St. Gallen, Switzerland). X and Y coordinates for webs were input to ArcView version 3.2a (Environmental Systems Research Institute Inc. 2000) where we calculated the nearest-neighbour distances using the Nearest Features version 3.7a extension (Jenness Enterprises 2004), and then plotted a histogram depicting the distribution of the nearest-neighbour distances using Systat version 10.2 (Systat Software Inc. 2002). The distribution of webs varied throughout both outcrops, but it was common to find refugia next to one another with the edges of cob webs overlapping. Also, we had an instance of two females retreating into the same refuge when disturbed, suggesting that some females may share webs. Although the mating status of wild females was unknown, some webs containing egg sacs were adjacent to webs without egg sacs, suggesting that the distribution of female mating status also varied spatially. The mean (±SD) nearest-neighbour distance for webs was 0.50 ± 0.36 for the first site and 0.72 ± 0.50 for the second site (Figs. 1C, 1D).

Female *L. hesperus* are most active between dusk and dawn, when they leave the refuge to settle on the outer web. During this time females clean, repair, and expand the web, thereby likely refreshing any existing web-borne chemicals. While females inhabit a web throughout their lives, males abandon their juvenile webs at sexual maturity and wander in search of a potential mate. This wandering phase is likely to result in high mortality for males (e.g., Gwynne 1987; Andrade 2003).
Female genitalia consist of paired independent sperm storage organs (spermathecae), each of which is inseminated via a separate opening and a long coiled duct. Males have corresponding coiled structures (emboli) that are located on paired anterior appendages (the pedipalps). Each embolus is inserted in one spermatheca via the coiled duct at copulation. Recent work on congeners (*L. hasselti*, *L. revivensis*) suggests that the male’s embolus tip must reach inside the spermatheca for insemination to be successful (Berendonck and Greven 2000; Snow 2003).

**Field experiment**

We examined whether or not male *L. hesperus* were differentially attracted to webs of conspecific females by placing empty webs in the field in a randomized block design that included four treatments: a control, webs from northern and southern populations of *L. hesperus*, and webs from the heterospecific *L. hasselti*.

We used webs built by female *L. hesperus* that were descended from black widow spiders collected in British Columbia (*n* = 15) and in Arizona (*n* = 15) to determine whether males can distinguish between different populations of *L. hesperus*, and used webs built by female *L. hasselti* that were descended from black widow spiders collected in Perth, Western Australia (*n* = 15), to determine whether males can distinguish between webs of different species.
females were from outbred lines that had been reared in the laboratory for at least two generations. Females were used approximately 6 months after maturity, were virgins at the time of web construction, and had no prior direct contact with males. Each female was placed in a screen cage (10 cm x 10 cm x 7.5 cm) in the laboratory in Toronto and was given 17 days to build a web (females began web construction within 24 h). Cages were shipped overnight to Kamloops where we removed the females and used the cages immediately. Before being placed in the field, each cage was surrounded on all sides by a 2.5 cm wide sticky strip cut from an insect glue trap.

We placed cages at 15 marked trap sites (“blocks”). Each trap site had four treatments: one web from each spider group (L. hesperus from British Columbia; L. hesperus from Arizona; L. hasselti) and one control. Trap locations were spread over both outcrops (eight covering a distance of 70 m in the first outcrop and seven covering a distance of 60 m in the second outcrop) and were in a row approximately in the centre of each outcrop, parallel to its long axis. Deviation from ideal locations was due to variation in terrain and vegetation, which made it impossible to place traps in some areas (Figs. 1A, 1B). At each trap site, we marked a 1 m² with a randomly determined orientation. A single cage (with glue trap frame) from each spider group was then randomly placed at each of three vertices. At the fourth vertex, we placed an empty glue trap frame as a control. Our traps were placed near naturally occurring female webs, so interweb distances were comparable with those found naturally on the outcrops (Figs. 1A, 1B), and the minimum distance between experimental cages (1 m) was within 1 or 2 SDs of the mean nearest-neighbour distances found in natural webs in the second and first sites, respectively (Figs. 1C, 1D). Two of the traps had to be moved away from the centre of the outcrop during the study since they were damaged by yellow-bellied marmots, Marmota flaviventris (Audubon and Bachman, 1841), on the first night. Males were attracted to both of these moved traps, so data from these traps were included in our analyses.

Each trap was checked daily at approximately 1100 PST for 3 days. Any males found on the glue strips or on the cages were collected. On the 3rd day, all the cages and glue strips were collected at 1400, and any other males found at this time were also collected. We then placed six empty cages (no webs) with sticky strips at six randomly chosen trap sites as an additional control and checked them over the following 3 days.

**Laboratory matings**

We conducted controlled laboratory matings to determine whether males could successfully mate with conspecifics from different populations. Laboratory-reared male L. hesperus from lines established with black widow spiders collected in British Columbia were paired with laboratory-reared conspecific females from a population established with black widow spiders collected in Arizona. Laboratory-reared males from Arizona were also paired with laboratory-reared females from British Columbia (interpopulation matings). We also carried out control matings within each population (intrapopulation matings). Five matings were carried out in each group for a total of 20 matings.

Females were placed in clear Rubbermaid® containers (35 cm x 30 cm x 15 cm) on wood doweling and allowed to build a web for at least 5 days. Males and females were fed prior to trials to ensure that variation in hunger level did not affect mating success. Trials began when a male was placed on a female’s web and were terminated after a maximum of 8 h, when a male had inserted both pedipalps (paired copulatory organs) or when a female knocked the courting male off the web. All mating trials were video recorded using Panasonic BP-330 cameras with Navitar Zoom 7000 zoom lenses.

For each trial, we examined (i) male courtship behaviour, which was defined as the presence or absence of the six stereotyped courtship behaviours that were described in Ross and Smith (1979); (ii) female receptivity, which was defined as whether the female vibrated her abdomen (Ross and Smith 1979); and (iii) male mating success, which was determined by examining the number of insertions of the pedipalps. If the male mated successfully, we fed females twice a week for 2 months to determine whether females produced any viable egg sacs.

Finally, we examined male pedipalps to determine whether there was a difference between the two populations of L. hesperus in embolus length or coil number. Since male and female genitalic morphology are closely matched in the genus Latrodectus (e.g., Kaston 1970; Berendownck and Greven 2000), we thought this might give insight into observed differences in fertilization success of males from the two populations (see Results). We stretched the embolus away from the palp using fine forceps and took digital photographs of the embolus under a dissecting microscope. We then measured embolus length on digital photographs using Image Tools version 3.0 (UTHSCSA 2002).

**Statistics**

We used a replicated goodness-of-fit test (G statistic) to determine whether there were differences between the number of males attracted to different cages, since this test (i) pooled data across days into a single value and (ii) also treated each day as a replicate that allowed the probabilities in each day to be combined into a single statistical test (Sokal and Rohlf 1995). We then used a χ² test for post hoc analyses between populations. For laboratory matings, we compared mating success and offspring production for interversus intra-population matings. We tested all data for normality and used nonparametric statistics where applicable. All values are expressed as mean ± SE.

**Results**

**Field experiment**

A total of 47 males were caught during the 3-day study (Table 1). No male L. hesperus were attracted to empty cages or to the glue traps alone (controls), although the glue traps did catch a variety of other ground-walking invertebrates (e.g., crickets, ants, beetles, Lepidoptera larvae). We removed control cages (zeros) from the analysis to avoid biasing the goodness-of-fit test towards significance. More than 50% of the captured male L. hesperus were on cages containing the web of females from the British Columbia population of L. hesperus (Table 1). Significantly more
males were attracted to the cages of conspecific females from the same population than to cages with the web of females from Arizona or from female *L. hasselti*. This is true whether data are pooled across days or whether each day is used as a replicate (Table 2). In addition, twice as many males were collected from cages filled with the web of heterospecific females (*L. hasselti*, 30% of males) than the web of conspecific females from the Arizona population (15% of males), although this difference was not significant ($\chi^2 = 2.33$, $p = 0.127$). Twenty-nine of the 47 captured males (62%) were found in, or on the cages, rather than on the sticky strips. The distribution of males attracted to the different black widow spider groups was similar for males caught on sticky strips and those caught inside cages (Table 1). The relative attractiveness of the different treatment webs was similar each day (heterogeneity test, $p > 0.05$). The total number of males caught each day decreased with time, although not significantly ($\chi^2 = 1.269$, $p = 0.53$).

### Laboratory matings

The stereotyped behaviours outlined in Ross and Smith (1979) were performed by all males except for four (three of which are from interpopulation matings); all of which led to unsuccessful matings (i.e., two of these males were knocked off the web and a third was killed). Eight of 10 males from intrapopulation pairings and 5 of 10 males from interpopulation pairings copulated. This difference was not statistically significant (one-tailed Fisher’s exact test $= 2.03$, $p = 0.16$). In general, we found no behavioural differences between inter- and intra-populational matings, but we had low power for our analyses because there were relatively few successful matings. For example, the number of copulations did not differ significantly between inter- and intrapopulation matings (one-tailed Fisher’s exact test $= 5.49$, $p = 0.06$). We observed female vibrations in 8 of 20 trials, although this behaviour did not occur more often in successful matings (one-tailed Fisher’s exact test $= 0.60$, $p = 0.44$) or in intrapopulation matings (one-tailed Fisher’s exact test $= 0.84$, $p = 0.36$). However, intrapopulation matings were significantly more likely to result in viable egg sacs than were interpopulation matings so that 50% of females (4/8) mated to males from the same population produced egg sacs, whereas none of the females mated to males from a different population produced egg sacs (0/5) (one-tailed Fisher’s exact test $= 4.649$, $p = 0.05$).

### Discussion

Our results strongly suggest that male *L. hesperus* use airborne pheromones released from webs to locate conspecific female webs in nature. No male black widow spiders were found on control cages, although other invertebrates were captured on these glue traps. There were no visual or tactile differences among web-filled cages, and females were removed so that males could not get cues from them directly. Nevertheless, large numbers of males were attracted to web-filled cages (Table 1). Moreover, males were found disproportionately on webs built by conspecific females from the
same geographic population, suggesting that airborne pheromones provided males with information which they could use to discriminate among webs of even closely related females. Since all females used in this study had been laboratory-reared on a common diet, this effect could not be due to environmental or diet-based effects on the chemical content of the web. Rather, this is likely to reflect heritable variation in the composition of the pheromone produced by female L. hesperus as a function of the species and its population of origin.

Since about half of the males were caught on or in the cages (Table 1), it is possible that some males entered cages without getting stuck on the sticky traps, were able to sample female webs directly, then left the traps again. Some of these males would likely get stuck as they exited the cages, but some might have exited successfully. If this were the case, the variation in attraction might be partly based on the assessment of web-bound rather than airborne pheromones. However, several lines of evidence support the conclusion that the distribution of males across female webs is due to attraction by airborne pheromones. First, all males found on sticky traps were oriented toward the cages, suggesting that they were trapped when first approaching the cage. No males were found in the opposite orientation, as would be expected if they entered cages initially then got stuck on exiting. Second, the distribution of males attracted across the black widow spider groups was similar whether we pooled all data, examined only males caught on the sticky strips, or examined only those found inside cages (Table 1). Third, males of the closely related L. hasselti remain on the webs of virgin females for days, even if there is no female present, so it is likely that males would arrive on a female’s web and remain there for the few hours before we collected them (M.C.B. Andrade, unpublished data).

Our study adds to the few studies demonstrating the importance of airborne pheromones in mate attraction in spiders (see Searcy et al. 1999; Papke et al. 2001). First, males of some species cut and cover portions of the web of a potential mate as soon as they arrive on the web, and this apparently limits the release of attractive volatiles that might attract rivals (Watson 1986; Schulz and Toft 1993). Second, some field studies report differential attraction of male spiders to the webs of virgin females compared with previously mated or immature females, suggesting the importance of cues that are effective at a distance (Robinson and Robinson 1980; Pollard et al. 1987; Anava and Lubin 1993). However, in the latter observational studies, females were present in webs or males were in direct contact with webs, so it was unclear whether these effects were due to differential male attraction to the web or variation in male departure from webs after exposure to contact pheromones or females.

Previous studies of spiders from the genus Latrodectus examined the effect of female contact pheromones on male courtship behaviour and showed that males initiate courtship on the webs of conspecific and heterospecific females (e.g., L. rivivensis, L. hesperus, L. mactans; Ross and Smith 1979, Anava and Lubin 1993). Additionally, Kaston (1970) was able to successfully mate L. hesperus with L. mactans, although only 3 of 27 attempts were successful and no egg sacs were produced. In Kaston’s (1970) study, the intensity of courtship was highest with conspecifics, suggesting that contact pheromones may also contribute to genetic isolation of these species. However, these studies also led to speculation that heterospecific copulations might be common in nature. Since matings with heterospecifics may be costly to males (aggression from heterospecific females (Kavale 1986) or no offspring produced (Kaston 1970) after risky mate searching), this has led to questions about how males avoid these potentially costly interactions. Our study suggests that, even in areas where two Latrodectus species overlap (e.g., L. hesperus and L. mactans in Texas; Levi 1959), most males could accurately locate the webs of conspecifics using airborne pheromones. Although we did not directly compare sympatric species, the discrimination of conspecific females from different populations suggests that males are able to make fine-grained decisions using pheromones. If this is true, there may be relatively weak selection for males to discriminate females based on contact pheromones that elicit courtship behaviour, which would explain the observation of heterospecific courtship in these spiders (Kaston 1970). However, contact pheromones could serve as late protection from wasted courtship efforts, since males do sometimes incorrectly identify females using long-distance pheromones (e.g., Table 1). This function may be reflected in the variation in courtship intensity elicited by heterospecific versus conspecific contact pheromones (Kaston 1970). Further field studies using different species whose ranges overlap (e.g., L. hesperus and L. mactans) would be helpful in determining whether this level of discrimination by males was common.
Perhaps the most interesting result of our study is the equal preference by males for webs built by heterospecific female *L. hasselti* and webs built by conspecific females from a different population. Pheromones are typically complex blends of chemicals (Prouvost et al. 1999) and female spiders are known to produce multiple pheromones that overlap between species (Trabalon et al. 1997). Thus, male *L. hesperus* may be attracted to webs of *L. hasselti* because of common chemical elements in the pheromones of the two species. Since there is no possibility of natural mis-matings between *L. hasselti* (Western Australia) and *L. hesperus* (western North America), there will not have been selection on males to discriminate cues produced by these females.

In comparison, although black widow spiders from northern and southern populations of *L. hesperus* are also unlikely to interact directly, because the species is distributed across western North America (from British Columbia to southern Texas; Levi 1959) and because juveniles disperse by ballooning (Foelix 1982), it is possible that individuals from adjacent populations may interact throughout the north–south axis. Although results from our interpopulation matings should be treated with caution as a result of the small sample size, they suggest that at least at the extremes of this north–south range populations of *L. hesperus* may be completely or partially reproductively isolated and may be incipient biological species. Since matings across populations result in a higher rate of reproductive failure than matings within populations, selection would favour males that discriminate against mates from other geographical regions. Our work suggests that such discrimination could be mediated by differential response to female pheromones detected prior to arrival at a web, which would minimize the cost of mate searching for males. The evolution of differences in female pheromones and male response in these two populations may not require long time periods; e.g., in moths, where the genetic basis of pheromone production and reception are well known, a single gene mutation can drastically alter the female pheromone, and male responses to pheromones are heritable and can be altered through selection in the laboratory (Roelofs et al. 1987; Evenden. et al. 2002). Further research comparing fitness effects of inter- and intra-population matings, relative male attraction, and heritability of differences in attractiveness are necessary to determine if divergence in airborne pheromones is coincident with incipient speciation in populations of *L. hesperus*.

Divergence in morphological traits could be responsible for the observed failure of interpopulation matings in *L. hesperus* owing to the physical incompatibility between populations. In addition, the observed population differences in embolus length (Fig. 2) could be important, as this affects the location of ejaculation. Longer emboli can reach into the female’s sperm storage organ during mating. In the case of males from British Columbia mating with females from Arizona, the male’s relatively short embolus might result in ejaculation in the insemination tubules rather than in the organ itself, which reduces fertilization success in congeners (Berendonck and Greven 2000; Snow 2003).

We also found a difference in body size between populations, with males from the southern population being significantly larger than those from the northern population (Fig. 2) despite the common laboratory-rearing environment. This suggests the intriguing possibility that differential selection on male body size in northern and southern populations has resulted in allometric changes in male copulatory organs, which could directly affect inter-population mating success (Panhuis et al. 2001; Schluter 2001). Future work will examine variation in ecological and social factors affecting selection on male body size and embolus length across the population range of *L. hesperus*, and test whether this variation may be leading to parapatric speciation.

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**References**


