

**Web reduction behaviour and the chemical
communication system of the western black
widow, *Latrodectus hesperus***

by

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B.Sc., Queen's University, 2008

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science

in the
Department of Biological Sciences
Faculty of Science

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Fall 2014

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Abstract

In spiders, pheromones mediate intersexual attraction, courtship behaviour, and mate recognition. I studied the chemical communication system of the western black widow spider, *Latrodectus hesperus*. Females attract males to their webs with silk-borne sex pheromones. Contact with the silk elicits courtship behaviour by males. Often, the courting male engages in web reduction behaviour, during which he excises and bundles up sections of the web and wraps them with his own silk. Using analytical chemistry, and behavioural tests in both the laboratory and the field, I investigated (1) the molecular structure and function of the female's contact sex pheromone, and (2) the function of web reduction behaviour. A silk-borne contact pheromone elicits web reduction behaviour, and a serine derivative similar to the pheromone of congeneric *L. hasselti* is one constituent of the multi-component pheromone. Web reduction decreases web attractiveness to rival males in the field, thereby limiting male-male competition.

Keywords: animal communication; behaviour; pheromones; spiders; silk

For Sam, who introduced me to spiders.

Acknowledgements

I gratefully acknowledge the Tsawout First Nation for granting permission to do field work on their beautiful land, and the Capital Regional District for granting permits for spider collection at Island View Beach.

I thank my supervisor Gerhard Gries for his endless enthusiasm and support, for always being available to talk through issues big and small, and for believing in me especially when I did not.

I thank my committee members Maydianne Andrade and Bernie Roitberg for their excellent guidance and helpful suggestions that have improved this work and my writing immensely.

I thank Robb Bennett for agreeing to examine my thesis, and for being an inspirational spider advocate.

I thank all of my labmates for advice, support, and friendship during my degree. Special thanks go to Bekka Brodie, Antonia Musso, Eloise Rowland, Chelsea Eby, and Mike Hrabar. I also thank my pseudolabmates Gwylim Blackburn, Tanya Stemberger, and Mike Boers for much needed moral support during the writing of my thesis.

I am very grateful to all the undergraduates that volunteered their time and energy to help with maintenance of spiders and laboratory and/or field work: Susan Chen, Chloe Gerak, Devin Kirk, Maïa Smith, and Elaine Wu.

I especially thank Samantha Vibert for her mentorship and friendship over the past four years. None of this work would have been possible without her support and insightful guidance.

I thank Sean McCann for being my best friend and collaborator, and for assistance with uncountable aspects of this work, including but not limited to photography, videography, field work, experimental design, and manuscript editing.

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Latrodectus hesperus female (left) and male (right) on the female's tangle-web

Chapter 1.

The role of male silk in spider courtship and sexual communication

1.1. Abstract

Spiders communicate via chemical, acoustic, tactile, and visual signals in various contexts including courtship and mating. All spiders produce silk, and even in non-web building spiders silk is intimately tied to sexual communication and interactions. Silk provides a transmission channel for male vibratory courtship signals, and webs and draglines provide a substrate for female sex pheromones. Behavioural observations indicate that many male spiders deposit silk during sexual interactions, but the function of the silk has rarely been investigated. Evidence is beginning to accumulate suggesting that silk-borne male pheromones may also be important in spider communication. This review will summarize the current knowledge of silk use by male spiders in the context of courtship and mating. I argue that ritualised silk deposition by males during courtship including 'bridal veils', silk-wrapped nuptial gifts, and web modification may all provide males a means of placing their pheromones in close proximity to the female's chemoreceptors. Such a mechanism of direct delivery of sex pheromones that function in stimulating females to mate, inducing catalepsis, or providing information about male quality would be advantageous for male spiders that often risk death from aggressive females during courtship and copulation. Future work focussed on the function of male silk will allow us to test specific hypotheses about inter-sexual signalling and thereby gain a deeper understanding of the complex chemical communication systems of spiders.

1.2. Introduction

1.2.1. Sexual communication in spiders

Spiders use chemical, acoustic, tactile, and (in some cases) visual signals in various contexts including courtship and sexual communication (reviewed in Uhl & Elias, 2011). Spider courtship and mating behaviour vary considerably among taxa, and may include communication with complex and/or multimodal signals. In general, spider sexual communication signals may function in mate attraction, recognition and assessment, orientation, stimulation of mating behaviour, and suppression of predatory behaviour (Schneider and Andrade 2011).

Because all spiders are predators (but see Meehan et al. 2009), courting individuals (usually males) may face a risk of sexual cannibalism. Behaviours that are thought to minimize this risk include opportunistic mating with feeding or moulting females (Fromhage & Schneider, 2004; Robinson & Robinson, 1980), mate binding (Zhang et al., 2011), and nuptial gift giving (Costa-Schmidt and Machado 2012). Females often enter a cataleptic or quiescent state during courtship, which may be the result of male vibratory, chemical and/or tactile signals (e.g., Becker et al., 2005; Aisenberg et al., 2008; Zhang et al., 2011; Ferretti et al., 2013).

1.2.2. Spider silk and sexual communication

Spider silk is intimately tied to courtship behaviour and sexual communication in several ways. Behavioural evidence suggests that the production of silk-borne pheromones by females is widespread in spiders (Schulz 2004, Gaskett 2007), as is vibratory communication between mating partners, with the transmission channel often being the female's silk (Elias and Mason 2010). Because spider pheromones and vibratory signaling have recently been reviewed (Elias and Mason 2010, Uhl and Elias 2011, Schulz 2013), here I will only briefly summarize silk use by females. Silk use by males in the context of sexual communication, however, has been infrequently reported, and its function has rarely been investigated experimentally (except, e.g. Zhang et al., 2011; Brum et al. 2011). Here I review male silk use during mating behaviour, but I will not discuss male silk use in sperm web construction, as this occurs prior to sexual interactions in most spider taxa (Foelix 2011). I will argue that male courtship behaviours

that include silk deposition may facilitate the delivery of silk-borne pheromones or chemical cues that function in inter- and/or intra-sexual communication into close proximity with the sense organs of their receivers.

1.2.3. A brief introduction to spider silk

Silk use is ubiquitous in spiders despite their varied lifestyles, from burrow dwellers to wandering hunters to aerial web-builders (Craig 2003, Foelix 2011). Different kinds of silk glands produce silk that is specialized for particular functions such as dragline silk used for 'lifelines' and dispersal via 'ballooning' or rappelling (ampullate glands), nest and egg-sac (cocoon) construction (aciniform and tubiliform glands), and of course prey capture (multiple glands) (Kaston 1964, Brunetta and Craig 2010, Foelix 2011). The Mesothelae and Mygalomorphae use silk that lines underground burrows and bind soil and vegetation into shelters. They generally capture prey by waiting at the entrance to a burrow or retreat; some expand the area over which they can detect prey using silk 'trip lines' and some even build sticky sheet webs attached to substrate (Craig 2003). The Araneomorphae, with more silk types at their disposal, build webs that range from simple structures to complex orbs. Amaurobiids build tubular retreats with threads radiating from the entrance, and dictynids lay down networks of capture threads on leaves. Agelenids and linyphiids build different kinds of horizontal sheet webs, while theridiids build tangle webs with loose and irregular 'sheets' from which gum-footed trapping threads extend. Orb-webs of different types are built by spiders in the Araneidae, Tetragnathidae, Nephilidae, Uloboridae, and Theridiosomatidae. Araneomorph hunting spiders in the Ctenidae, Lycosidae, Salticidae, Oxyopidae, Pisauridae, and Trechaleidae have secondarily reverted from web building to wandering (Foelix 2011).

1.3. Female silk and sexual communication

Even in wandering spiders, female silk in the form of draglines provides information to mate-searching males. Draglines can act as tacto-chemical 'signposts' that convey information about the location and identity of the signaller, and webs or silk outside burrow entrances provide the stage for vibratory courtship displays by males.

1.3.1. Female silk: substrate for pheromones

Evidence for the existence of sex pheromones is widespread among the Araneae, but to date the molecular structure of pheromones has been determined for species in only six families (Pholcidae, Araneidae, Linyphiidae, Theridiidae, Agelenidae, and Ctenidae) (reviewed in Schulz, 2004, 2013; Gaskett, 2007; Uhl & Elias, 2011; Trabalon, 2013; Uhl, 2013). Most work has focused on female sex pheromones and silk-borne chemical cues, which function in mate attraction, recognition and assessment, as well as eliciting courtship behaviour. By using silk as a substrate for pheromones, spiders are capable of continuously emitting chemical signals without having to actively release them from the glands where they are produced (Schulz, 1997). Silk pheromones may be synthesized in silk glands or be applied to silk after secretion from cuticular glands (reviewed in Schulz, 2004; Trabalon, 2013). Spiders detect chemical signals with sensory hairs and pit organs that are most concentrated on the distal segments of the legs and pedipalps, but also occur on the mouthparts (Vallet et al. 1998, Tichy et al. 2001, Jiao et al. 2010, Foelix 2011, Trabalon, 2013). However, both the biosynthesis of chemical signals and chemoreception (especially olfaction) in spiders are still poorly understood.

Spiders are generally solitary and thus face the challenge to attract or find mates. Volatile, airborne sex pheromones typically attract mates at long range, and may also reveal information about the identity and quality of the signaler, which is usually the female (Gaskett 2007; Uhl and Elias 2011; Uhl 2013). Volatile, attractive sex pheromones have been identified from the bodies and/or silk of females in only three species: *Argiope bruennichi* (Araneidae: Chinta et al. 2010), *Agelenopsis aperta* (Agelenidae: Papke et al. 2001), and *Pholcus beijingensis* (Pholcidae: Xiao et al. 2009). Behavioural studies with *Latrodectus* spp. (Theridiidae) indicate that the volatile, silk-borne female pheromone allows males to discriminate between females of different age, mating status, body condition, and population of origin (Kasumovic and Andrade 2004, Andrade and Kasumovic 2005, MacLeod and Andrade 2014).

Contact sex pheromones have been identified from the silk of female spiders in four species: *Linyphia triangularis* (Linyphiidae: Schulz and Toft 1993), *Latrodectus hasselti* (Theridiidae: Jerhot et al. 2010), *Tegenaria atrica* (Agelenidae: Prouvost et al. 1999), and *Cupiennius salei* (Ctenidae: Papke et al. 2000). Silk-borne contact

pheromones elicit male searching and courtship behaviour, and provide information about female identity, mating status, receptivity, and quality (Gaskett 2007, Uhl and Elias 2011, Tralalon 2013).

1.3.2. Female silk: substrate for transmission of vibratory signals

Spiders are extremely sensitive to vibrations, which they detect via receptors on their legs (Barth 1982). Spiders that build webs or snares, or simply extend the silk lining of their burrow, can essentially expand their field of sensory perception, creating their own specialized signaling environments (Elias and Mason 2010, Krafft and Cookson 2012). The silk in these contexts transmits vibrations both from prey and courting males. Substrate-borne vibrations are extremely important for spiders (Barth 2002, Elias and Mason 2010) but we know very little about the transmission properties of silk. The types of vibratory signals in spiders include percussion, stridulation, and tremulation (reviewed in Uhl and Elias 2011). Vibratory courtship signals through webs have been recorded in only a few studies (Masters and Markl 1981, Masters 1984, Suter 1984, Naftilan 1999, Wignall and Herberstein 2013, Vibert et al. 2014).

1.4. Male silk and sexual communication

Evidence for male silk-borne pheromones and chemical cues is scarce but comes from diverse and distantly related families (Fig. 1). Given that female silk pheromones are common and widespread among spiders, it is plausible that male silk pheromones are also common, but thus far under-studied. In this review, I will highlight the widespread use of silk by male spiders in sexual interactions, specifically during web modification, mate-binding, and the formation of nuptial gifts. I will argue that these behaviours appear ideal for delivering silk-borne chemical signals into close proximity with the chemo-receptors of females, and should be targets of future research to improve our understanding of chemical communication in spiders.

1.4.1. Male silk: substrate for sex pheromones

Only one male spider pheromone has been identified to date, an aphrodisiac isolated from the bodies of *Pholcus beijingensis* (Pholcidae) males (Xiao et al. 2010).

However, behavioural evidence demonstrates or supports the existence of silk-borne male pheromones in six families (Fig. 1; Table 4). Like female pheromones, these putative male pheromones have diverse functions. Contact with male silk elicits courtship behaviour in female *L. hesperus* (Ross and Smith 1979). A silk-borne pheromone induces quiescence in female *Eratigena agrestis* (Agelenidae; S. Vibert, unpublished data). Tactile and/or chemical information on male silk facilitates orientation in female *Tegenaria domestica* (Agelenidae) and *Coleotes terrestris* (Amaurobiidae; Roland, 1983). Airborne pheromones from the bodies and silk of male *Scytodes* sp. (Scytodidae) and *Evarcha culicivora* (Salticidae) function in mate recognition and mate choice (Koh et al. 2009, Cross and Jackson 2009).

1.4.2. Male silk: cues for assessing competitive conditions

Male silk functions not only in inter-sexual communication but may also be important for information transfer between males (Table 5), including assessment of male-male competition. Airborne chemical cues from *Latrodectus hasselti* (Theridiidae) males and/or their silk provide information about the competitive environment and trigger shifts in development in other males (Kasumovic and Andrade 2006). Male *Nephila senegalensis* (Nephilidae) use silk cues left behind by rival males to choose which females' webs to visit, avoiding those a previous male visited, irrespective of the female's quality (Schneider et al. 2011). Male courtship behaviour is inhibited by a pheromone that can be extracted with methanol from the silk of *Scgizocosa ocreata* (Lycosidae) males (Ayyagari and Tietjen 1987), and *Frontinella pyramitela* (Linyphiidae) males respond to compounds on male cuticle with aggressive behaviour (Suter et al. 1987).

1.5. Male silk use during courtship

Spiders produce dragline silk as they move about (Foelix 2011). Therefore, it is likely that males in many species deposit silk during courtship and mating behaviour. Nonetheless, explicit descriptions of silk deposition by males in the context of courtship are rare (but see Barrantes and Ramirez 2013; Table 2), except for three specific types of male behaviour: (1) web reduction or modification with silk addition, (2) application of silk 'bridal veils' to females, and (3) presenting silk-wrapped 'nuptial gifts' to females. The

function of these types of behaviour have not been thoroughly studied, but the limited evidence to date suggests that all may function in sexual communication, implying that male silk is more important in this context than is currently known.

1.5.1. Male silk: web modification and silk addition

Courtship in web-building spiders generally takes place on the female's web. During courtship, modification of existing web architecture and/or addition of male silk occur in the families Linyphiidae, Theridiidae, Araneidae, Nephilidae, Dictynidae, Psecridae, Filistatidae, and Agelenidae (Table 1).

Web reduction behaviour

Web reduction appears to be a common aspect of courtship in both the Linyphiidae and Theridiidae (Table 1). This behaviour entails cutting the silken threads of the female's web, bundling up sections of web, and adding male silk (first described by van Helsdingen 1965; Fig. 2). The frequency at which this behaviour occurs – both within and among species – is variable, as is the extent to which the web is destroyed (see Table 1). Female aggression may affect whether or not web reduction takes place during courtship in *Florinda coccinea* (Linyphiidae; Robertson and Adler 1994). Similarly, the incidence of web reduction in *L. hesperus* is correlated with the degree of female aggressiveness (Scott et al. 2012). Males of *Neriene litigiosa* (Linyphiidae) reduce the webs of only highly receptive virgin females that have added pheromones to their silk; webs of newly matured and mated females do not elicit web reduction (Watson 1986). Males of *L. hesperus* more often reduce the webs of virgin than mated females, and of well fed than starved females, based on silk cues alone (C. Scott, unpubl. data).

The function of web reduction has been studied experimentally only with *N. litigiosa* (Watson 1986) and *L. hesperus* (Chapter 3). In both species, reduced webs are less attractive to males than intact webs, indicating that males that engage in web reduction decrease the probability of their long (several hours) courtship displays being interrupted by rival males. The effect of web reduction is presumably long lasting, because mated females rebuild their webs without pheromones, so web-reducing males also benefit by decreasing the probability of sperm competition (Watson 1986). For *N. litigiosa*, Watson (1986) argues that web reduction limits female silk pheromone

emission by decreasing the exposed silk surface area. Webs reduced by *L. hesperus* males, with capture web area decreased by about 50% on average, attracted half as many males as webs with 50% of the silk experimentally removed. The hypothesis that male silk deposited during web reduction acts as an anti-aphrodisiac and deters other males from entering a web was not supported in experiments with *L. hesperus* (Chapter 3). However, further work is required to determine the mechanism(s) by which web reduction decreases web attractiveness.

Curtailling pheromone emission from female webs and thereby reducing male-male competition is just one of several non-mutually exclusive functions of web reduction that have been hypothesized. Isolating the female from extraneous vibrations, such as those produced by prey or other males arriving at the web, is another potential function of web reduction (Rovner 1968, Lubin 1986). Restricting the mobility of the female, or reducing the area of web that a male must defend from competitors, have also been suggested (van Helsdingen 1965; Ross & Smith 1979; Breene & Sweet 1985). Vibrations associated with cutting silk lines could transmit information to the female or attract her attention (Forster 1995, Berendonck 2003). Furthermore, changing the web architecture might improve the transmission of vibratory courtship signals (Berendonck 2003). Web reduction behaviour (including depositing large amounts of silk) may be an energetically costly activity that provides the female with information about male quality (Anava and Lubin 1993, Harari et al. 2009). Pheromones on male silk might also stimulate the female to mate, or induce catalepsis (Ross and Smith 1979, Anava and Lubin 1993). For *Florinda coccinea* (Linyphiidae), Willey-Robertson and Adler (1994) argue that web reduction is not likely to reduce female pheromone emission, because males do not remove the entire web, and females occasionally deposit fresh silk during courtship. Instead, the authors propose that semiochemicals bound to the male's silk render the female's pheromone unattractive, or signal their presence to rivals. During web reduction males deposit large amounts of silk that they wrap extensively around dismantled sections of the web. The bundled silk is often left in close proximity to the female (Anava and Lubin 1993) rather than discarded. By forming ropes and balls of female silk, males produce a substrate for depositing their own silk, which may provide a concentrated area for emission of silk pheromones directed at the female and/or approaching males.

Web modification: mating threads and webs

The courtship behaviour of orb-weavers (Araneidae and Nephilidae) is grouped into three types (Robinson and Robinson (1980)). Type A courtship occurs on the female's web. Type B courtship begins on the web, with the male at some point cutting a hole in the web close to the hub and constructing a mating thread across the hole, on which vibratory courtship and copulation take place. Type C courtship does not involve any web cutting. Instead, the male constructs a mating thread that he attaches to the periphery of the web, and he performs vibratory courtship on this thread, onto which the female eventually moves and where copulation takes place. During all three types of courtship, the male adds silk to the female's web, either in the form of the mating thread or dragline silk that he produces as he moves around on the female's web. Only Type B courtship entails destruction of the female's web (Table 1).

Male cobweb weavers in the family Theridiidae commonly construct mating threads during courtship on the female's web (Table 1). The male installs a silk line and then plucks and vibrates on it until the female eventually moves onto the thread where copulation occurs (Knoflach 2004). In some species, the male reinforces the thread several times, or he constructs a larger area of threads referred to as a mating web, which is used in a similar way to mating threads (Knoflach 2004). In a few species, the male cuts some of the female's threads, but in general, theridiids other than those in the genus *Latrodectus* seem to modify the web by adding their own silk rather than excising some of the female's silk.

During courtship in a *Fecenia sp.* (Psecridae), the male cuts away most of the web, leaving only a single thread on which courtship and mating proceed (Robinson and Lubin 1979). Males of *Dictyna arundinacea* (Dictynidae) cut a hole in the web and construct a 'canopy' of their own threads on which they mate (Locket 1926). Males of the social spider *Achaeraena wau* (Theridiidae) build courtship 'arenas' in their communal webs by cutting out small areas of the barrier web threads and by laying down one or more of their own threads, on which courtship occurs (Lubin 1986). These threads are considered functionally equivalent to the mating threads of araneids (Lubin 1986).

In species that construct a mating thread either within or outside the female's web (see Table 2), the male may avoid an aggressive female by cutting the silk line

(Robinson & Lubin, 1979; Robinson & Robinson, 1980). In general, mating threads and webs most likely function to direct vibrations to the female and/or exclude extraneous vibrations (Robinson and Robinson 1980). In those species where the female moves on to the mating thread or mating web and remains there during copulation, her tarsi will necessarily be in contact with the male's silk. Thus, it is plausible that contact chemical signals on the male's silk are directly transmitted to the female's chemoreceptors.

Silk deposition without web modification

Upon contact with the silk of a virgin female, but not a mated female, males of *Kukulcania hibernalis* (Filistatidae) pull swaths of threads from their spinnerets with their last pair of legs and deposit them on her web (Barrantes and Ramirez 2013). Given that males invest considerable time and a large amount of silk in this behaviour, it is assumed to be costly. Barrantes and Ramirez (2013) speculate that silk deposition in combination with abdomen vibration function to reduce female aggressiveness in *K. hibernalis*.

'Abdomen wagging' behaviour by males occurs during the courtship of several agelenids (Table 1; Galasso 2012). Courting males of *Eratigena agrestis* (Agelenidae) use similar behaviour to deposit silk which they periodically anchor to the female's web (S. Vibert, personal communication). Most likely, other agelenids that perform abdomen wagging are also depositing silk throughout courtship. Preventing *E. agrestis* males from spinning silk during courtship severely hinders their ability to induce quiescence in females, providing strong evidence for a silk-borne pheromone (S. Vibert, unpublished results).

With mating threads and other versions of web modification, male spiders add their own silk to the female's webs and via that silk may deliver their pheromones into close proximity to or direct contact with the female. If pheromone is indeed present on male silk, the female may sense it through contact chemoreceptors on the distal segments of her legs and palps as she moves around her web, or she may sense the volatile pheromone component(s) through olfactory-type sensilla. It is not yet clear whether the tip pore sensilla that most likely function as contact chemoreceptors in spiders also function in olfaction, or if distinct receptors detect volatile compounds (Uhl 2013).

Web modification and silk addition: inferences and future study

Potential functions of web modification include directing vibrations toward the female and/or excluding extraneous vibrations, reducing female movement or the territory a male must defend against rivals, inhibiting female pheromone emission, and delivery of male silk-borne pheromones. The possibility that compounds in the male's silk contribute to decreased attractiveness of reduced webs or function in inter-sexual communication during courtship should be explicitly tested in future studies. Evidence from an agelenid indicates that silk-borne pheromone is important for inducing quiescence in females (S. Vibert, unpublished data). Further studies of agelenids, and of species (such as *K. hibernalis*) in other families which deposit large amounts of silk during courtship may be especially useful in determining the function of male silk and identifying associated pheromones.

1.5.2. Male silk: the 'bridal veil' or mate-binding behaviour

The term 'bridal veil' was coined by Bristowe (1931) in his description of the mating behaviour of *Xysticus cristatus* and *X. krakatuensis* (Thomisidae). Other descriptions for bridal veil spinning behaviour include 'tying', 'mate-binding', 'silk-binding' and 'trussing'. If any form of silk deposition by a male on a female's body during courtship or copulation is considered a bridal veil, this behaviour has been reported for at least 13 families (Table 2). There are several types of bridal veil, and this tying behaviour is more or less stereotyped and occurs in different contexts depending on the species (Table 2).

Males of *Xysticus* spp. (Thomisidae; Bristowe 1931, 1958), *Schizocosa malitiosa* (Lycosidae; Aisenberg et al. 2008), and *Dolomedes triton* (Pisauridae; Carico 1993) deposit silk over the female's first two pairs of legs and anchor the silk to substrate. In *Pisaurina mira* (Pisauridae; Bruce and Carico 1988) and *Oxyopes schenkeli* (Oxyopidae; Preston-Mafham 1999), both mates hang from a dragline below a plant, and the male deposits the 'veil' on the first two or three pairs of the female's legs while spinning her around. Males systematically deposit a ring-like veil around the female's legs as she stands on the ground in *Homalonychus selenopoides* and *H. theologus* (Homalonychidae; Domínguez and Jiménez 2005, Alvarado-Castro and Jiménez 2011), or as the female hangs from her mating web in *Thalassius spinosissimus* (Pisauridae;

Sierwald 1988). Males of *Ancylometes bogotensis* (Ctenidae) wrap the distal segments of the female's legs first with an outer ring of silk, and then add a second inner ring around the patellae (Merrett 1988). Complex, extensive silk-binding behaviour has also been described for species in the Nephilidae and Araneidae (see Table 2). In these families the diminutive males move around on the dorsum of the female, spinning silk between the bases of her legs, over her cephalothorax, and between her cephalothorax and abdomen.

A light or partial wrapping of the female with silk has been described for species in the Dictynidae, Tetragnathidae and Theridiidae (Table 2). Here, silk wrapping behaviour occurs on the female's web. Males also deposit some silk across females in *Tibellus oblongus* (Philodromidae; Preston-Mafham 1999), and *Tegenaria agrestis* (Agelenidae; S. Vibert personal communication). These types of veiling behaviours seem to be less ritualised than the types described above, and the wrapping is less extensive. Furthermore, while mate-binding is an obligate courtship behaviour in at least one nephilid species (Kuntner et al. 2009), in theridiids there is variation in occurrence within as well as across species. For example, veiling took place in about 33% of courtship observations in *Latrodectus revivensis* (Anava and Lubin 1993), in 50% of *Steatoda bipunctata* pairs (Knoflach 2004), and only occasionally in *Steatoda grossa* (C. Scott personal observation).

Males of *Cupiennius coccineus* (Ctenidae) normally do not use bridal veils during courtship, but in staged matings between *C. coccineus* males and *C. salei* females (which are slightly larger than *C. coccineus* females), some males deposited silk on the females (Schmitt 1992). Of the three males that spun silk around the female's legs, two males mated successfully and one was consumed. Schmitt (1992) suggested the 'veil' in this case might be part of prey-capture behaviour that has spilled over into the context of mating due to conflicting cues from the unusually large (i.e., possibly prey-like) female. Alternatively, the veil is indeed part of the courtship repertoire of *Cupiennius* males but is used exclusively for especially large and potentially dangerous females. That the veil had not previously been observed could be attributed to size-matched pairs typically being chosen for laboratory mating trials (Schmitt 1992).

The most commonly hypothesized function of bridal veils is the reduction of female aggression, thus minimizing the risk of cannibalism. There has been some

debate as to whether the veil physically restrains the female. Most descriptions indicate that females are quickly and easily able to break free of their silken bonds, making this interpretation unlikely, but some authors argue that the brief moments of struggling free from the veil may provide the male with just enough time to escape from a potentially cannibalistic female (Breene and Sweet 1985, Bruce and Carico 1988). Alternatively, chemical or tactile signals on the veil rather than physical constraints may help suppress a female's predatory or aggressive tendencies. The male's silk may be impregnated with pheromone that inhibits movement of the female so that she remains in a cataleptic state during copulation (Ross and Smith 1979, Aisenberg et al. 2008, Preston-Mafham 1999). Such a silk-borne pheromone could also provide the female with information about the male's identity or quality (Ross and Smith 1979, Anava and Lubin 1993). Incapacitating the female's sensory hairs by direct contact with the veil (Lopez 1978), or stimulation of the female (Robinson and Robinson 1973; Preston-Mafham 1999), are other suggested mechanisms of action or functions of the bridal veil. Pheromone-impregnated silk may also trigger physiological changes that prepare the female for mating (Preston-Mafham 1999). As with web reduction, the male's silk could also function to repel rival males, possibly via pheromones (Aisenberg et al. 2008).

The function of the bridal veil has been investigated experimentally in a single study with *Nephila pilipes* (Zhang et al. 2011). Here, some males mated successfully without depositing any silk, but mate binding behaviour following an interrupted first copulation attempt invariably resulted in a second copulation. In contrast, those males that did not spin a bridal veil were more likely to be cannibalized without obtaining a second copulation. Recording the behaviour of males with occluded spinnerets, and of females with ablated or occluded tactile and chemical receptors, revealed that tactile cues associated with tying behaviour may be most important, with chemical cues playing a secondary role. Zhang et al. (2011) suggest that pheromone-producing glands are likely located near the spinnerets, and conclude that the bridal veil in *N. pilipes* reduces the risk of sexual cannibalism and allows males to overcome resistance of females to repeated copulations.

In many species, silk deposition by males seems to target the dorsal surface of the distal segments of the female's legs, usually the first two or three pairs (Table 2). Thus, the silk comes in contact with areas where chemoreceptors are concentrated

(Aisenberg et al. 2008). If males do have silk-borne pheromones, deposition of silk on the female's body might be an efficient way to bring the pheromone in direct contact with the female's chemoreceptors. It is also possible that tactile cues from direct contact with male silk provide the female with information.

Bridal veils: summary

The function of mate-binding behaviour is not well understood. It could have evolved several times independently in species with especially large or aggressive females (Schmitt 1992). Silk wrapping in the context of mating may have evolved from silk wrapping in prey capture (Schmitt 1992), which minimizes the risk of injury from dangerous prey (Foelix 2011). Lopez (1978) suggests that the bridal veil silk may be aciniform, like prey-capture silk, but this has yet to be investigated. The bridal veil as a method for direct delivery of male silk-borne pheromone (and/or tactile signals) to female sensory receptors is a hypothesis that warrants further investigation. Experimental studies of silk-binding behaviour will be critical to understanding its function.

1.5.3. Silk-wrapped nuptial gifts

Nuptial gifts, items transferred from a male to a female that help secure mating (reviewed by Vahed et al. 1998, Vahed 2007), are rare in spiders. The types of gifts given by spiders include the male's body, glandular secretions from the male's cephalothorax, and silk-wrapped prey (reviewed in Albo et al. 2013b). Here I will focus on silk-wrapped prey items as nuptial gifts, which have been reported for several species in the closely related families Trechaleidae and Pisauridae (Table 3). Anecdotal reports suggest that males of the theridiid *Argyrodes elevatus* (Uetz et al. 2010, Cobbold and Su 2010) might also present silk-wrapped nuptial gifts (Table 3), but this phenomenon requires further investigation.

The function of silk-wrapped nuptial gifts has been studied most carefully in *Pisaura mirabilis* (Pisauridae) and *Paratrechalea ornata* (Trechaleidae). Female silk cues (probably pheromones) elicit courtship and gift construction in males of both *P. ornata* (Albo et al., 2009) and *P. mirabilis* (Albo et al., 2011). However, female silk is not required to elicit gift-wrapping by *P. mirabilis* males, who sometimes prepare nuptial gifts before they encounter a female or her draglines (Lang, 1996; Albo et al., 2011). When a

male *P. mirabilis* or *P. ornata* finds a female, he presents his gift by holding it in his chelicerae in a characteristic display. If the female accepts his gift, she grasps it with her chelicerae and copulation ensues while she is feeding on the gift. Nuptial gifts in *P. mirabilis* represent male signals that have evolved by sexual selection through cryptic female choice for sperm storage (Albo et al. 2013a). Males that provide nuptial gifts to mates have higher mating success because they copulate longer and transfer more sperm (Albo et al. 2013a). In *P. ornata*, nuptial gifts also increase mating success and result in longer copulations, increased paternity, and accelerated oviposition by females (Albo & Costa, 2010).

Most *P. ornata* males that encounter females while carrying gifts wrap them with silk after contact with the female (Albo & Costa, 2010). Adding silk after a female's rejection likely increases attractiveness of the gift (Brum et al. 2011). Visual signals alone do not affect gift acceptance, but ether-extractable compounds specific to nuptial gift silk elicit female acceptance of filter paper 'gifts' (Brum et al. 2011). Females more often accepted gifts wrapped by males than gifts wrapped with silk experimentally reeled from males' spinnerets, suggesting that males control the type of silk they use or the compounds they add to the silk during gift construction (Brum et al. 2011). Thus, there is strong evidence that pheromones on male silk stimulate females to accept gifts, thereby increasing mating success of males. Females responded similarly to silk extract and prey extracts, implying that the pheromone either resembles prey cues and exploits the female's foraging response, or comprises unrelated compounds that elicit the same response, the acceptance of and feeding on the gift (Brum et al. 2011). Wrapping low-quality gifts in pheromone-laden silk may be a strategy of males that minimizes the costs of providing a gift while maintaining its attractiveness, because females do accept an empty prey carcass (worthless gift) if it is wrapped in silk (Albo and Costa 2010, Brum et al. 2011). The pheromone on silk-wrapped nuptial gifts may provide information about a male's quality; perhaps this is an honest signal, whereas the gift itself does not necessarily honestly indicate prey capture ability.

Unlike in *P. ornata*, evidence for the importance of pheromones in *P. mirabilis* is more direct. Silk-wrapping affords *P. mirabilis* males greater control over their gift. Because they can maintain their grip on the silk, they lower the risk of the female stealing the gift without mating, and the round shape of the wrapped gift facilitates

access to the female's genitalia for copulation thus increasing their mating success (Andersen et al., 2008). Silk wrapping is not strictly required for *P. mirabilis* females to accept nuptial gifts (Bilde et al. 2007). However, if males attempt to mate and are rejected, they often add silk to their gifts, and then go on to mate successfully, suggesting that sex pheromone on the male's silk affects female acceptance of the gift or otherwise manipulates female reproductive behaviour (Bilde et al. 2007). Female *P. mirabilis* feed longer on gifts wrapped with more silk, and most males already carrying wrapped prey wrap it again after encountering a female (Lang 1996). The extent of silk-wrapping during gift construction depends on male condition in *P. mirabilis*, with better-condition males adding more silk, and thus may be an honest signal of male quality (Albo et al., 2011). However, silk may also function in hiding worthless gift contents. A male that presents a silk-wrapped gift containing a prey carcass or plant material instead of prey can obtain a short copulation that ends as soon as the female detects that there is no prey inside the silk (Albo et al. 2011). The thick silk wrapping of the nuptial gift does not itself provide a source of protein to the female (Nitzsche 1988 as cited by Albo et al. 2013b) but may contain phagostimulants (Albo et al. 2013b), a hypothesis that should be investigated.

Silk use during mating in a ray spider: A nuptial gift with web modification and possible bridal veil

The very small ray spider *Theridiosoma gemmosum* (Theridiosomatidae) has an apparently unique behaviour during mating that seems to combine elements of web modification, mate-binding, and nuptial gift giving. Between the 16 to 48 copulations that may take place during a single mating, males pay out silk from their spinnerets, which is collected and ingested by the female (Hajer and Řeháková 2011). *T. gemmosum* males spin mating threads between plants and the hub of the female's conical orb-web. "A tangle of threads spun by the male during its movements around the sitting female," (Hajer and Řeháková 2011) may also be an example of a bridal veil.

Mature males do not feed, thus the amount of silk a male can spin before or during mating depends on his ability to capture prey as a juvenile. Because silk is energetically costly to produce (Craig 2003), the amount of silk a male produces may be an honest signal of his quality. Hajer and Řeháková (2011) consider the silk the male spins during copulation to be a gift of nutrients, because araneoids can recycle silk

proteins by consuming silk (Craig 2003). Neither this function nor any other has yet been tested.

Although described as dragline silk, the silk *T. gemmosum* males feed to females may serve a specific feeding function, as appears to be the case for the silk used to wrap nuptial gifts in a trechaleid (Brum et al. 2011). It would be interesting to test whether males can mate successfully without depositing silk. I speculate that the male silk added near or on the female prior to mating could induce her receptivity or quiescence, and that the additional silk the male spins between bouts of copulation could 'refresh' that signal. Before and while the female consumes the silk, she handles it with her first pair of legs. Thus, both her mouthparts and distal segments of her first pair of legs and pedipalps, which bear contact chemoreceptors (Trabalon 2013), almost certainly come into direct contact with the male's silk throughout copulation. The ray spider system provides an excellent opportunity to further study the functions and mechanisms of male silk deposition during sexual interactions in spiders.

Nuptial gifts: summary

By providing silk or a silk-wrapped gift to a female, a male may deliver pheromone directly to the contact chemoreceptors on the female's palps and chelicerae. Because the female feeds on the gift throughout copulation, this may provide a mechanism for continuous pheromone transmission. Many spiders mate opportunistically with females engaged in feeding (e.g., Fromhage and Schneider 2004); the presentation of nuptial gifts may be a refinement of this mating strategy. Silk-borne male pheromones have been implicated in the gift-giving system of *P. ornata*, but the mechanism remains unclear (Brum et al. 2011). Similarly, phagostimulants have been hypothesized to be present on male silk in *P. mirabilis* (Bilde et al. 2007), but this hypothesis has not yet been tested. The type of silk appears to be important. It may be produced by the aciniform glands, like the swathing silk used by some spiders for prey-capture (Lopez 1987). Future studies should compare the chemical properties of silk used to wrap a nuptial gift to those of other silk types. This will facilitate the identification of putative semiochemicals on the silk and study of their specific function.

1.6. Conclusion

The bulk of our knowledge of spider mating behaviour comes from extensive study of a small number of families (Araneidae, Ctenidae, Linyphiidae, Lycosidae, Pholcidae, Salticidae, Theridiidae; Schneider and Andrade 2011). Nonetheless, the widespread occurrence of silk deposition during courtship and mating (Figure 1) suggests an important function of male silk. That the silk is invariably deposited in close proximity to the female, and often in direct contact with her chemoreceptors, lends support to the hypothesis that male silk is a substrate for semiochemicals. The limited experimental evidence thus far on the function of male silk during courtship behaviour suggests that silk plays an important role in both inter- and intra-sexual communication. Behavioural studies already support the existence of male silk-borne pheromones in several spider taxa. The species identified in this review should become targets for experimental studies on the function of male silk during mating and for identification of putative silk-borne sex pheromones. Male silk may also play an important role in intra-sexual communication by providing cues that allow rival males to assess the competitive environment both before and during encounters with a female. Male silk deposited on a female's body or web during sexual interactions should be investigated for the presence of chemical cues that facilitate avoidance of competition and/or anti-aphrodisiac pheromones that render females less receptive or attractive to rival males. Male chemical signals and cues are an under-studied aspect of spider communication that will provide rich opportunities for future research.

To better understand male silk use in courtship and communication, the functional roles of both the silk itself and the behaviours associated with its deposition must be investigated. Preventing males from depositing silk during courtship by occluding their spinnerets with wax or glue is a good technique for investigating the function of male silk. Ablating female chemoreceptors may also be useful in determining the function and importance of chemical signals (e.g. Zhang et al., 2011). Testing the responses of males to the silk of rival males in the context of mate-searching and mate choice (e.g. Schneider et al. 2011) will allow us to determine the function of silk in intra-sexual communication. In species where behavioural evidence indicates the presence of a male silk-borne pheromone, pheromone identification should be pursued. Comparative pheromone analyses of male and female silk may be especially fruitful in those species

in which the female pheromone is known. Tichy et al. (2001) have obtained electrophysiological responses to volatile components from tarsal chemoreceptors in *Cupiennius salei*, and 'electrolegograms' have already been developed for whip spiders (Amblipygi; Hebets and Chapman 2000). As our knowledge of spider chemoreception improves, we should strive to develop an analog of the gas chromatographic-electroantennographic detection (GC-EAD) system previously invented for analyses of insect pheromone (Struble and Arn 1984). This analog technique would entail using a spider's chemoreceptive appendage in place of an insect antenna as a sensor to determine the volatiles that elicit sensory responses. Such a technique would allow rapid screening for potential pheromones in extracts from spider silk or cuticle. Future studies should also attempt to determine the glandular origins of silks and of associated semiochemicals that males produce during courtship and mating behaviour.

Acknowledgements

I thank Sean McCann for providing the photograph and editing the manuscript, and Samantha Vibert for providing the video, inspiring the topic of the review, and many helpful comments on the manuscript. Funding was provided by a Simon Fraser University Graduate Fellowship to CS.

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Table 1.1 Spider taxa in which males modify the female's web by adding silk and/or reducing her web. Family numbers are according to Platnick (2014). Y = yes, N = no, P = probable, *n* = number of mating interactions observed. Where data are available, the specific behaviour is described in brackets, as is the % of males that engage Spider taxa in which males deposit silk 'bridal veils' onto the female during courtship. Family numbers are according to Platnick (2014). in that behaviour, with a superscript indicating the reference specific to these data where necessary.

#	Taxon	Addition of silk	Web reduction	Reference
21	Filistatidae			
	<i>Kukulcania hibernalis</i>	Y (83%; <i>n</i> = 6)	N	Barrantes and Ramirez 2013
56	Theridiidae			
	<i>Achaeranea tepidarorium</i>	Y (web-spinning; <i>n</i> = 3)	N	Knoflach 2004
	<i>Achaeranea wau</i>	Y (small mating arena)	Y (small area reduced)	Lubin 1986
	<i>Anelosimus aulicus</i>	Y (mating thread; <i>n</i> = 6)	Y (hole cut for mating thread)	Knoflach 2004
	<i>Argyrodes antipodiana</i>	Y	Y	Whitehouse and Jackson 1994
	<i>Argyrodes argyroides</i>	Y (web-spinning; 14%; <i>n</i> = 7)	Y	Knoflach 2004
	<i>Dipoena melanogaster</i>	Y (mating web)	N	Knoflach 2004
	<i>Echinotheridion gibberosum</i>	Y (mating web)	N	Knoflach 2004
	<i>Enoplognatha afrodite</i>	Y (mating web; <i>n</i> = 4)	N	Knoflach 2004
	<i>Enoplognatha latimana</i>	Y (mating web; <i>n</i> = 1)	N	Knoflach 2004
	<i>Enoplognatha ovata</i>	Y (mating web; <i>n</i> = 5)	N	Knoflach 2004
	<i>Enoplognatha diversa</i>	Y (mating web; <i>n</i> = 3)	N	Knoflach 2004
	<i>Enoplognatha macrochelis</i>	Y (mating web; <i>n</i> = 5)	N	Knoflach 2004
	<i>Enoplognatha quadripunctata</i>	Y (mating web; <i>n</i> = 2)	N	Knoflach 2004

Table 1.1 continued

#	Taxon	Addition of silk	Web reduction	Reference
	<i>Enoplognatha thoracica</i>	Y (mating web; $n = 1$)	N	Knoflach 2004
	<i>Latrodectus dahli</i>	Y (50%; $n = 2$)	N	Knoflach and van Harten 2002
	<i>Latrodectus geometricus</i>	Y	Y ('less commonly')	Segoli et al. 2008
	<i>Latrodectus hasselti</i>	Y	Y	Forster 1992, 1995
	<i>Latrodectus hesperus</i>	Y	Y (up to 50% of web; 58%; $n = 12^1$)	Ross and Smith 1979; 1Scott et al. 2012
	<i>Latrodectus mactans</i>	Y	Y	Breene and Sweet 1985
	<i>Latrodectus pallidus</i>	Y	Y	Harari et al. 2009
	<i>Latrodectus revivensis</i>	Y (69% of males)	Y (up to 50% of barrier web)	Anava and Lubin 1993
	<i>Meta segmentata</i>	Y (copulation thread)		Bristowe 1929
	<i>Paidiscura</i> sp.	Y (mating web)		Knoflach 2004
	<i>Steatoda bipunctata</i>	Y (mating web; $n = 3$)	Y (removed threads)	Knoflach 2004
	<i>Steatoda castanea</i>	Y (mating web; $n = 1$)		Knoflach 2004
	<i>Steatoda grossa</i>	Y ($n = 6$)	Y (>50% of web)	C. Scott unpublished data
		Y (mating web and/or web-spinning; $n = 4$)	N	Knoflach 2004
	<i>Steatoda paykulliana</i>	Y (silk-throwing; 66%; $n = 3$)	N	Knoflach 2004
	<i>Steatoda triangulosa</i>	Y (mating thread; $n = 4$)	Y (removed threads; 50%; $n = 4$)	Knoflach 2004
	<i>Theridion pallens</i>	Y	Y	Locket 1927
	<i>Theridion simile</i>	Y	N	Locket 1927
	<i>Theridion varians</i>	Y	Y	Locket 1927
	<i>Tidarren argo</i>	Y (mating web)	N	Knoflach 2004

Table 1.1 continued

#	Taxon	Addition of silk	Web reduction	Reference
	<i>Tidarren cuneolatum</i>	Y (mating web)	N	Knoflach 2004
65	Linyphiidae			
	<i>Florinda coccinea</i>	Y	Y (part of web; 75%; $n = 20$)	Willey Robertson and Adler 1994
	<i>Lepthyphantes leprosus</i>	Y	Y (90-100% of web; 45%; $n = 29$)	van Helsdingen 1965
	<i>Linyphia triangularis</i>	Y	Y (part or all of web ² ; 68%; $n = 60$ ³)	² Rovner 1968 ³ Weldingh et al. 2011
	<i>Neriere litigiosa</i>	Y	Y (large portions of web; 28%; $n = 50$)	Watson 1986
	<i>Pitohyphantes phrygianus</i>	unknown	Y (web reduced to a small wad; $n = 18$) ⁴	⁴ Stålhandske and Gunnarsson 1996, Gunnarsson et al. 2004
67	Nephilidae			
	<i>Nephila</i> spp. (grp. A)	Y (dragline silk)	N	Robinson and Robinson 1980
	<i>Herennia</i> spp. (grp. A)	Y (dragline silk)	N	Robinson and Robinson 1980
68	Araneidae			
	<i>Argiope aemula</i>	Y ('miniweb' within web)	N	Robinson and Robinson 1980
	<i>Argiope</i> spp. (grp. A and B)	Y (deposition on the hub)	N	Robinson and Robinson 1980
	<i>Argiope</i> spp. (grp. B)	Y (mating thread)	Y (small hole)	Robinson and Robinson 1980
	<i>Argiope keyserlingi</i>	Y (mating thread)	Y (small hole)	Wignall and Herberstein 2013
	Various spp. (grp. C)	Y (mating thread)	N	Robinson and Robinson 1980

Table 1.1 continued

#	Taxon	Addition of silk	Web reduction	Reference
76	Psechridae			
	<i>Fecenia</i> sp.	N	Y (most of web, leaving single thread in situ; $n = 1$)	Robinson and Lubin 1979
79	Agelenidae			
	<i>Agelenopsis actuosa</i>	P (abdomen waggle)	N	Galasso 2012
	<i>Agelenopsis aperta</i>	P (abdomen waggle)	N	Singer et al. 2000
	<i>Agelenopsis aleenae</i>	P (abdomen waggle)	N	Galasso 2012
	<i>Agelenopsis aperta</i>	P (abdomen waggle)	N	Galasso 2012
	<i>Agelenopsis emertoni</i>	P (abdomen waggle)	N	Galasso 2012
	<i>Agelenopsis kastoni</i>	P (abdomen waggle)	N	Galasso 2012
	<i>Agelenopsis naevia</i>	P (abdomen waggle)	N	Galasso 2012
	<i>Agelenopsis oklahoma</i>	P (abdomen waggle)	N	Galasso 2012
	<i>Agelenopsis pennsylvanica</i>	P (abdomen waggle)	N	Galasso 2012
	<i>Agelenopsis potteri</i>	P (abdomen waggle)	N	Galasso 2012
	<i>Agelenopsis spatula</i>	P (abdomen waggle)	N	Galasso 2012
	<i>Agelenopsis utahana</i>	P (abdomen waggle)	N	Galasso 2012
	<i>Barronopsis texana</i>	P (abdomen waggle)	N	Galasso 2012
	<i>Eratigena agrestis</i>	Y (depositing silk)	N	Vibert et al. 2014

Table 1.1 continued

#	Taxon	Addition of silk	Web reduction	Reference
85	Dictynidae <i>Dictyna arundinacea</i>	Y (small 'canopy')	Y (small hole)	Locket 1926

Table 1.2 Spider taxa in which males deposit silk ‘bridal veils’ onto the female during courtship. Family numbers are according to Platnick (2014).

#	Taxon	Context	Type of veil	Reference
52	Uloboridae <i>Uloborus</i> sp.	female's web	unknown	Gerhardt 1933 (as cited by Berendonck 2003)
56	Theridiidae <i>Euryopis episinoides</i>	female's web	some silk on female	Knoflach 2004
	<i>Latrodectus geometricus</i>	female's web	some silk on legs and body	Knoflach and van Harten 2002; Segoli et al. 2008
	<i>Latrodectus hasselti</i>	female's web	some silk on legs and body	Forster 1992
	<i>Latrodectus hesperus</i>	female's web	some silk on legs and body	Ross and Smith 1979; Kaston 1970; Herms et al. 1935; Scott et al. 2012
	<i>Latrodectus indistinctus</i>	female's web	some silk on legs and body	Smithers 1944
	<i>Latrodectus mactans</i>	female's web	some silk on legs and body	Breene and Sweet 1985
	<i>Latrodectus pallidus</i>	female's web	some silk on legs and body	Shulov 1940
	<i>Latrodectus revivensis</i>	female's web	some silk on legs and body	Anava and Lubin 1993
	<i>Latrodectus tredecimguttatus</i>	female's web	some silk on legs and body	Shulov 1940
	<i>Steatoda bipunctata</i>	female's web	some silk on female	Knoflach 2004
	<i>Steatoda grossa</i>	female's web	some silk on legs and body	C. Scott personal observation
	<i>Steatoda paykulliana</i>	female's web	some silk on legs and body	Knoflach 2004
	<i>Steatoda triangulosa</i>	female's web	some silk on female	Knoflach 2004

Table 1.2 continued

#	Taxon	Context	Type of veil	Reference
66	Tetragnathidae <i>Metellina segmentata</i>	female's web	female wrapped with fine silk	Bristowe 1929; Lopez 1987
67	Nephilidae <i>Herennia ornatissima</i> <i>Nephila pilipes</i>	female's web female's web	silk on and around abdomen silk on carapace, legs and abdomen (extensive)	Robinson and Robinson 1980 Kuntner et al. 2009; Robinson and Robinson 1980
68	Araneidae <i>Argiope aemula</i>	female's web	silk on carapace, legs and abdomen (extensive)	Robinson and Robinson 1980
69	Lycosidae <i>Schizocosa malitiosa</i>	substrate	legs I & II tied to substrate	Aisenberg et al. 2008
71	Pisauridae <i>Dolomedes triton</i> <i>Pisaurina mira</i> <i>Thalassius spinosissimus</i>	substrate hanging on dragline female's mating web	legs I & II tied to substrate silk spun around legs I & II silk ring around patellae	Carico 1993 Bruce and Carico 1988 Sierwald 1988
72	Oxyopidae <i>Oxyopes schenkeli</i>	hanging on dragline	silk spun around legs I, II, & III	Preston-Mafham 1999

Table 1.2 continued

#	Taxon	Context	Type of veil	Reference
78	Ctenidae <i>Cupiennius coccineus</i>	substrate	some silk on legs	Schmitt 1992
79	Agelenidae <i>Eratigena agrestis</i>	female's web	some silk on legs and carapace	S. Vibert unpublished data
85	Dictynidae <i>Dictyna volucripes</i>	female's web	some silk on female	Starr 1988
102	Homalonychidae <i>Homalonychus selenopoides</i> <i>Homalonychus theologus</i>	substrate substrate	silk ring around legs silk ring around legs	Alvarado-Castro and Jiménez 2011 Domínguez and Jiménez 2005
112	Philodromidae <i>Tibellus oblongus</i> <i>Tibellus</i> sp.	substrate substrate	some silk on female some silk on female	Preston-Mafham 1999 Platnick 1971
113	Thomisidae <i>Xysticus cristatus</i> <i>Xysticus krakatuensis</i>	substrate substrate	legs I & II tied to substrate legs I & II tied to substrate	Bristowe 1931; Bristowe 1958 Bristowe 1931

Table 1.3 Spider taxa in which males present females with silk-wrapped nuptial gifts. Family numbers are according to Platnick (2014).

#	Taxon	Type of gift	Reference
56	Theridiidae <i>Argyrodes elevatus</i>	spider lightly wrapped in silk stolen silk-wrapped prey	Cobbold and Su 2010 Uetz et al. 2010
57	Theridiosomatidae <i>Theridiosoma gemmosum</i>	silk	Hajer and Řeháková 2011
70	Trechaleidae <i>Paratrechalea azul</i> <i>Paratrechalea galianoe</i> <i>Paratrechalea ornata</i> <i>Trechalea amazonica</i> <i>Trechalea bucculenta</i>	silk-wrapped prey silk-wrapped prey silk-wrapped prey silk-wrapped prey silk-wrapped prey	Costa-Schmidt et al. 2008 Costa-Schmidt et al. 2008 Costa-Schmidt et al. 2008 Silva and Lise 2009 Silva 2005 (as cited by Silva and Lise 2009)
71	Pisauridae <i>Pisaura lama</i> <i>Pisaura mirabilis</i> <i>Parenethis fascigera</i>	silk-wrapped prey silk-wrapped prey silk-wrapped prey	Itakura 1993 (as cited by Costa-Schmidt et al. 2008) Bristowe and Lockett 1926; Bristowe 1958 Itakura 1998

Table 1.4 Spider taxa in which there is behavioural evidence for male pheromones. Family numbers are according to Platnick (2014). These families are also indicated in red in fig. 1.

#	Taxon	Source	Type	Female response	Reference
23	Scytodidae <i>Scytodes</i> sp.	body and silk	airborne	mate choice	Koh et al. 2009
29	Pholcidae <i>Pholcus beijingensis</i>	body	airborne	stimulates mating behaviour	Xiao et al. 2010
56	Theridiidae <i>Latrodectus hesperus</i>	silk	contact	courtship	Ross and Smith 1979
69	Lycosidae <i>Allocosa alticeps</i> <i>Allocosa brasiliensis</i> <i>Trochosa</i> sp.	body body silk	airborne airborne contact	courtship courtship mate recognition	Aisenberg et al. 2010 Aisenberg et al. 2010 Engelhardt 1964 (as cited by Uhl & Elias 2011)
79	Agelenidae <i>Agelenopsis aperta</i> <i>Eratigena agrestis</i> <i>Tegenaria domestica</i>	body silk silk	airborne unknown contact	quiescence/catalepsis quiescence/catalepsis orientation	Becker et al. 2005 S. Vibert unpublished data Roland 1984
86	Amaurobiidae <i>Coelotes terrestris</i>	silk	contact	orientation	Roland 1984

Table 1.4 continued

#	Taxon	Source	Type	Female response	Reference
114	Salticidae <i>Evarcha culicivora</i>	body and silk	airborne + contact	courtship and attraction/mate recognition	Cross and Jackson 2013

Table 1.5 Spider taxa in which there is behavioural evidence for males responding to silk cues of other males. Family numbers are according to Platnick (2014).

#	Taxon	Source	Type	Male response	Reference
56	Theridiidae <i>Latrodectus hasselti</i>	body and/or silk	airborne	shift in development	Kasumovic and Andrade 2006
65	Linyphiidae <i>Frontinella pyramitela</i>	silk cuticle	contact contact	positive geotaxis aggressive behaviour	Suter and Hirscheimer 1986 Suter et al. 1987
67	Nephilidae <i>Nephila sengalis</i>	silk	contact	avoidance/mate choice	Schneider et al. 2011
69	Lycosidae <i>Lycosa rabida</i> <i>Scgizocosa ocreata</i>	body silk	airborne airborne and contact	reduces exploratory behaviour inhibits courtship	Tietjen 1978 Ayyagari and Tietjen 1987

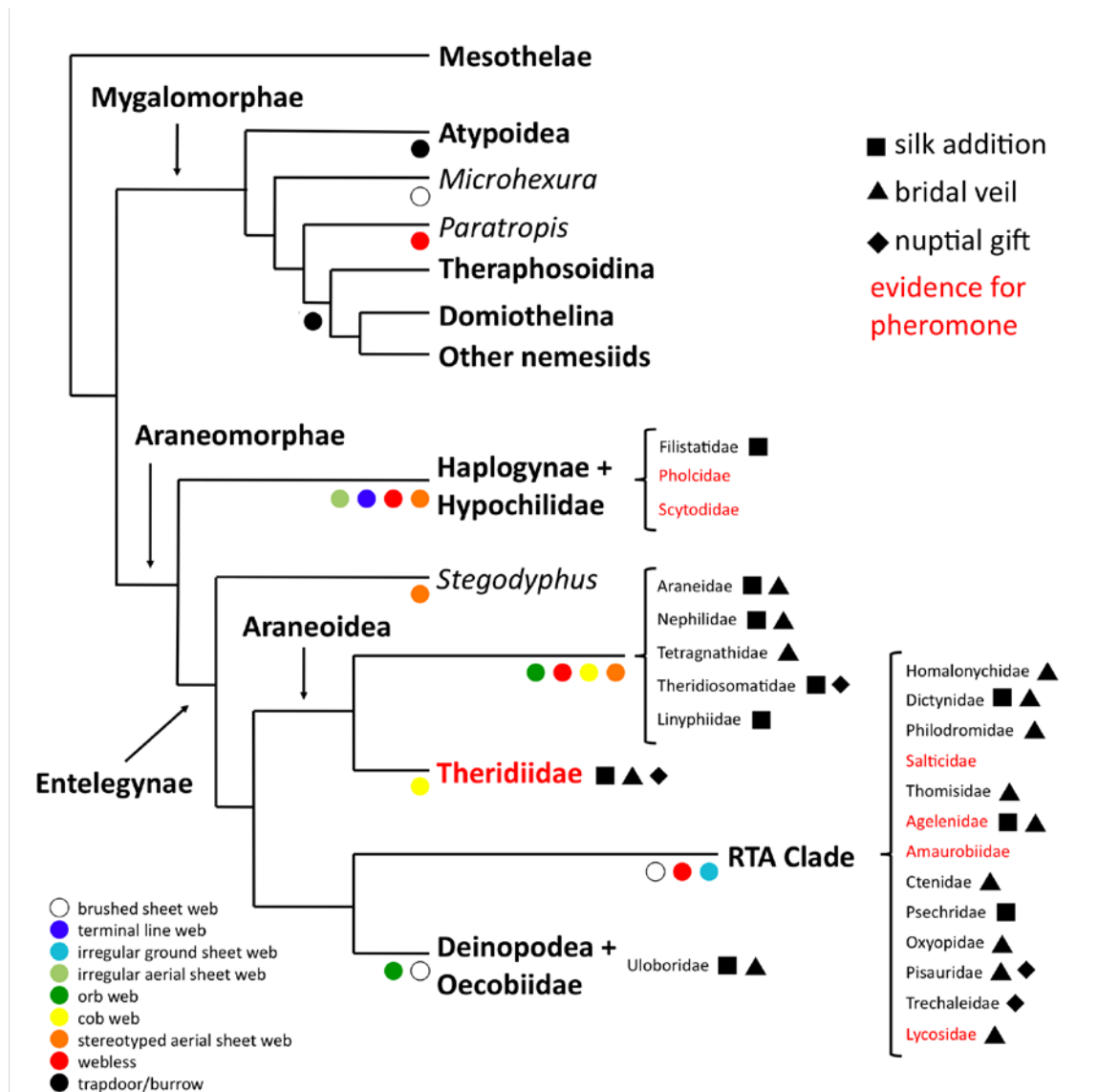


Figure 1.1 Phylogeny of Araneae showing web type (after Bond et al. 2014), indicating families in which there are records of bridal veils, nuptial gifts, or silk addition during courtship (see tables 1-3 for species names and references). Families in which there is evidence for male sex pheromones are indicated in red (see table 4 for species names and references)



Figure 1.2 A male black widow (*Latrodectus hesperus*) wraps a bundled section of a female's web with silk during web reduction behaviour, using his last pair of legs to pull the silk out of his spinnerets

Chapter 2.

***N*-3-Methylbutyryl-*O*-methylpropanoyl-*L*-serine methyl ester – pheromone component of western black widow females**

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This chapter has been submitted to Journal of Chemical Ecology.

2.1. Abstract

Chemical communication is common in spiders but few pheromones have been identified. Female widow spiders in the genus *Latrodectus* spin webs that disseminate an attractive sex pheromone, and contact pheromone on the silk elicits courtship behaviour by males. The methyl ester of *N*-3-methylbutyryl-*O*-(*S*)-2-methylbutyryl-*L*-serine is such a contact pheromone of the Australian redback spider *Latrodectus hasselti*. We predicted that the contact pheromone of congeneric *L. hesperus* resembles that of *L. hasselti*. We extracted the silk of virgin *L. hesperus* females with methanol, analyzed aliquots of silk extract by gas chromatography-mass spectrometry (GC-MS), and found evidence for *N*-3-methylbutyryl-*O*-methylpropanoyl-*L*-serine methyl ester (MB-MP-S), a lower homologue of the *L. hasselti* contact pheromone. We tested behavioural responses of *L. hesperus* males to test stimuli on T-shaped rods with the end sections of the horizontal arm enveloped in filter paper. Males spent 40% longer in contact with paper bearing female silk than blank paper, and 39% longer in contact with paper treated with silk extract than solvent controls. Contact with silk and silk extract induced courtship behaviour by 96% and 80% of males, respectively, indicating that there is a methanol-soluble courtship-eliciting contact pheromone on the silk. Males responded less strongly to synthetic MB-MP-S than to silk or silk extract. Paper impregnated with synthetic MB-MP-S (10 or 100 µg) induced courtship behaviour in 3-16% of males, and prompted males to stay 10-16% longer than on control paper. Our data support the

conclusion that MB-MP-S is part of a multi-component contact pheromone of *L. hesperus*.

2.2. Introduction

Chemical communication is common in spiders, but only few spider pheromones have been identified (Gaskett 2007; Schulz 2013). Spider sex pheromones on the silk or cuticle mediate attraction, mate recognition, and courtship behaviour (Uhl and Elias 2011). Generally, volatile airborne pheromones attract mates at long range, whereas contact pheromones elicit courtship, and may reveal information about the identity, quality, or mating history of the signaler, which is usually the female (Gaskett 2007). To date, female sex pheromones have been identified for only seven spider species in six families (Schulz 2013). Three of these pheromones are volatile and attract males. For example, (2*R*,3*S*)- and (2*S*,3*S*)-trimethyl methyl citrate at ratios of 6:1 to 25:1 comprise the attractive pheromone of virgin female orb-weaving wasp spiders, *Argiope bruennichi* (Araneidae)(Chinta et al. 2010). *N*-3-Methylbutyryl-*O*-(*S*)-2-methylbutyryl-*L*-serine methyl ester represents one of four known silk-borne contact pheromones; it is produced by virgin females of the tangle-web weaving Australian redback spider *Latrodectus hasselti* (Theridiidae) and elicits searching and courtship behaviours by males (Jerhot et al. 2010). 8-Methyl-2-nonanone exemplifies a bi-functional pheromone; it is produced by sexually receptive females of *Agelenopsis aperta* (Agelenidae) and both attracts males and induces courtship behaviour (Papke et al. 2001).

The attractiveness of volatile spider pheromones to conspecific males is typically tested in still-air olfactometers in the laboratory (e.g., Xiao et al. 2009) or in field-trapping experiments (e.g., Chinta et al. 2010). Both choice and no-choice bioassays allowing males to contact silk or pheromone-impregnated filter paper have been used to determine the behavioural responses of males to contact pheromones. For example, in no-choice experiments, *L. hasselti* males were placed on filter paper impregnated with synthetic pheromone or a solvent control and their behaviour was video recorded (Stoltz et al. 2007; Jerhot et al. 2010). Because specific courtship behaviours are difficult to observe in this type of bioassay, movement instead of courtship behaviour of males was used to assess pheromone activity.

Here we investigate the function and molecular structure of the sex pheromone of female western black widows, *Latrodectus hesperus*. As shown in field studies, volatile pheromone emanating from webs of conspecific females attracts *L. hesperus* males and allows them to discriminate between virgin and mated females (Kasumovic and Andrade 2004; MacLeod and Andrade 2014). When a male contacts a virgin female's silk, he engages in courtship behaviour that includes vibratory signaling and web reduction behaviour, during which he cuts the female's web and wraps sections of it with his own silk (Ross and Smith 1979; Scott et al. 2012). We do not yet know whether the same pheromone acts as both an airborne attractant and a contact courtship-releaser in this species or congeners. In *Linyphia triangulosa* (Linyphiidae), the volatile breakdown products of the web reduction-eliciting contact pheromone (3*R*,3*R'*)-3-hydroxybutyryloxybutyric acid on the silk are airborne attractants (Schulz and Toft 1993; Schulz 2013).

The sex pheromones of *Latrodectus* females may not be entirely species-specific. The webs of *L. mactans* females elicit courtship behaviour not only of conspecific males but also of *L. hesperus* males (Ross and Smith 1979). Furthermore, in a field experiment in British Columbia, *L. hesperus* males were attracted not only to empty webs of local conspecific females, but also to webs of *L. hasselti* females, even more so than to the webs of conspecific females from Arizona (Kasumovic and Andrade 2004). Several species in the genus *Linyphia* share the same contact pheromone that elicits web reduction behaviour but discriminate between the silk of con- and hetero-specifics, presumably based on additional silk-borne compounds (Schulz and Toft 1993; Schulz 2013).

We tested the hypotheses (1) that a silk-borne pheromone of female *L. hesperus* elicits short-range attraction of males and/or courtship behaviour, and (2) that the molecular structure of the pheromone resembles that of the *L. hasselti* pheromone.

2.3. Methods and Materials

2.3.1. Experimental Animals

All spiders in this study were offspring from mated females collected at Island View Beach, on the Saanich peninsula of Vancouver Island, British Columbia, Canada (48° 35' N, 123° 22' W, elevation 3–4 m). We reared spiders in the laboratory on a diet of house crickets (*Acheta domesticus*) and blow flies (*Lucilia sericata*), and kept spiders at 20–25 °C on a reversed 12L:12D photo regime to facilitate experimentation during their nocturnal activity period.

2.3.2. Collection of Silk for Behavioural Bioassays

We allowed female spiders to build webs for 72 h in wood-frame boxes (30 × 30 × 20 cm). These boxes were re-used for housing virgin females only, and between uses all silk and prey remains were removed and the boxes were wiped out with a damp cloth. We did not feed the spiders used for silk collection after placing them in boxes, so that no prey cues would be present on the silk. We then removed the silk from the box using glass pipettes and wrapped it around a filter paper envelope (see below; Fig. 1a), distributing it as evenly as possible along the length of the paper.

2.3.3. Collection of Silk for Chemical Analyses

We provided virgin female spiders (1–3 months post maturity) with equilateral, triangular prism glass frames (Fig. 1b) for web construction. To facilitate grip on substrate and to prevent spiders from leaving, we sandblasted the frames and placed them on a glass base surrounded by a moat of water. Following 72 h of web building, we collected the silk with clean glass pipettes, placed it in a 4-mL glass vial, and extracted the silk with methanol for at least 24 h. For chemical analyses, we combined extracts of 21 webs and concentrated the sample to a volume of ~200 µL. For behavioural tests, we combined extracts of at least eight webs for each batch, and diluted the silk extract to a volume of 40 µL per web.

2.3.4. Analyses of Silk Extracts

We analyzed aliquots of silk extract using a Saturn 2000 Ion Trap GC-MS operated in full-scan electron impact mode and fitted with a DB-5 GC-MS column (50 m × 0.25 mm i.d.), setting temperatures of the injector port and ion trap to 250 °C and 260 °C, respectively. We used helium as the carrier gas (35 cm s⁻¹) with the following temperature program: 100 °C for 5 min, 20 °C min⁻¹ increase until 280 °C, 280 °C for 20 min.

2.3.5. Syntheses

General methods and instrumentation for syntheses, a representative synthesis of ester-amide mixtures, and synthesis of *N*-3-methylbutyryl-*O*-methylpropanoyl-*L*-serine methyl ester (MB-MP-S) are reported in Online Resource 1.

2.3.6. General Design of Behavioural Experiments

We examined the responses of virgin male spiders (5–30 days post maturity) to test stimuli in two-choice experiments. We constructed T-shaped rods (henceforth ‘T-rods’; Fig. 1b) from pairs of bamboo skewers (25.5 cm; Bradshaw International Inc., Rancho Cucamonga CA, USA) joined with a piece of labeling tape (3 × 0.5 cm; Fisher Scientific, Ottawa ON, Canada). We secured the vertical arm of the T-rod to an inverted paper cup (Solo Cup Company, Lake Forest, IL, USA) filled with floral foam, and placed a piece of filter paper (2.5 × 2.5 cm; Whatman No. 1, Whatman International Ltd., Maidstone, Kent, UK) folded in half to form an envelope over each end section of the horizontal arm and stapled it ca. 3 mm from the edge. We used new T-rods and filter papers for each bioassay.

We applied a test stimulus to one filter paper and a control stimulus to the other (see Table 1), alternating the treatment side of the T-rod between tests. When we tested extract or synthetic MB-MP-S, we allowed the methanol solvent to evaporate before we introduced a male spider to the base of the T-rod. We scored each spider’s behaviour in real time using Jwatcher v1.0 (Blumstein et al. 2012), beginning when he reached the intersection of the ‘T’. We ran bioassays for 30 min (experiments 1 and 5) or 15 min (experiments 2-4), or until the spider descended from the T-rod on his dragline;

whichever came first. We used a cutoff of 15 min in experiments 2-4 because the results from experiments 1 and 5 were almost identical whether we analysed only the first 15 min or the entire 30 min. For each test, we recorded three behavioral response criteria: (1) first choice; (2) percent time spent on treatment and control stimuli; and (3) courtship behaviour.

For criterion 1 (first choice), we recorded the filter paper a male contacted first, predicting that it would be the paper treated with silk, silk extract, or synthetic MB-MP-S if the response was based on airborne cues.

For criterion 2 (percent time spent on treatment and control stimuli), we calculated the percentage of total time on the T-rod that spiders spent on each filter paper, predicting that males would spend more time on the paper treated with silk, silk extract, or synthetic MB-MP-S if silk has a courtship-eliciting contact pheromone.

For criterion 3 (courtship behaviour), we recorded whether or not the spider wrapped the treatment filter paper with silk, predicting that males would engage in silk-wrapping on filter papers treated with silk, silk extract, or synthetic MB-MP-S. 'Silk-wrapping' refers to the male pulling silk from his spinnerets with his last pair of legs and depositing it onto the female's web (Fig. 2a). During silk wrapping, the male adopts a distinctive posture with his abdomen raised relative to his cephalothorax, and with his last pair of legs he describes wide arcs as he pulls silk out of his spinnerets (Fig. 2b and Online Resource 2). This behaviour is a part of normal courtship sequences in this species, wherein the male wraps silk around a section of the female's web (web reduction behaviour; Ross & Smith 1979; Scott et al, 2012). We selected silk-wrapping as the response criterion for courtship behaviour because it is unmistakable in the context of our bioassay.

2.3.7. Specific Behavioural Experiments

In experiment 1, we tested the effect of female silk, silk extract, and synthetic MB-MP-S on responses of males (see Table 1 for summary). We tested the responses of the same 25 male spiders in each of four 2-choice tests presented in random order. Test stimuli were as follows: (i) the silk of a single virgin female's web (1 FW = the entire web of a single female) wrapped around filter paper vs. a blank paper control; (ii)

methanol extract of 1 female web equivalent (1 FWE = total silk extract of one web) vs. methanol control, (iii) synthetic MB-MP-S (10 μ g) vs. methanol control, and (iv) methanol control vs. methanol control. We ran the control vs. control test (iv) to ensure that there was no directional bias in the set-up.

To verify that the results for experiment 1 were not affected by males being repeatedly exposed to silk pheromone components, we ran additional tests. In each of experiments 2 and 3, we used the same treatments and controls as in (ii) and (iii) above, testing 39 naïve males that had never courted a female or been exposed to female silk extract or synthetic pheromone.

In experiment 4, we tested the effect of a higher (100 μ g) dose of synthetic MB-MP-S on responses of naïve males (Table 1). We gave 20 male spiders a choice between synthetic MB-MP-S (100 μ g) and a solvent control, predicting that the 100- μ g dose would elicit stronger responses from males than the 10- μ g dose in experiment 1 or 3. In spiders, 10-fold increases in pheromone dose are typically required to increase responses by a factor of two (e.g. Papke et al. 2001; Jerhot et al. 2010).

In experiment 5, we tested the effect of synthetic MB-MP-S admixed with silk extract on responses of a new group of naïve males (Table 1). We gave 34 male spiders a choice between silk extract (0.5 FWE) admixed with synthetic MB-MP-S (10 μ g) and silk extract alone (0.5 FWE), predicting that the former stimulus would elicit stronger responses from males because it contained a larger amount of the candidate pheromone in addition to other potential pheromone components present in the silk extract.

2.3.8. Statistical Analyses

For each T-rod test, we analyzed data with 2-tailed paired *t*-tests to determine whether males spent a greater percentage of time on the treatment filter paper than the control filter paper. For experiment 1, we analyzed data with ANOVA, using individual spiders as blocks, to determine whether the difference between the percentage of time males spent on the treatment filter paper relative to the control filter paper differed between tests. We followed the ANOVA with post-hoc tests for differences among

treatments, adjusting for multiple comparisons using the Tukey method. We ran all statistical tests in R 3.0.2 (R Core Team 2013).

2.4. Results

2.4.1. Pheromone Analysis

GC-MS analyses of silk extract of female *L. hesperus* revealed a quantitatively minor component (**A** in Fig. 3a) with a fragmentation pattern (Fig. 3b) closely resembling that of *N*-3-methylbutyryl-*O*-2-methylbutyryl-*L*-serine methyl ester (MB-MB-S), the contact pheromone of *L. hasselti* (Jerhot et al. 2010). Based on the retention index (RI) (Van den Dool and Kratz 1963) of **A** (1740) we concluded that it could be a lower homologue of the *L. hasselti* pheromone (RI: 1832). We therefore synthesized ester-amide mixtures, using *L*-serine hydrochloride, isobutyric and isovaleric acids as starting materials (Online Resource 1). One of 12 resulting compounds had retention and mass spectral characteristics identical to **A**. Its mass spectrum was indicative of *N*-3-methylbutyryl-*O*-methylpropanoyl-*L*-serine methyl ester (MB-MP-S), a lower homologue of the *L. hasselti* pheromone. Retention and mass spectral characteristics of synthetic MB-MP-S (synthesized according to Jerhot et al. 2010; Online Resource 1) were in complete agreement with those of **A**, confirming our structural assignment. We considered MB-MP-S a good candidate pheromone component for *L. hesperus* because it resembled the pheromone of female *L. hasselti* and it was present in minor amounts in extracts of *L. hasselti* silk (Jerhot et al. 2010), which might explain the attraction of *L. hesperus* males to female *L. hasselti* webs.

2.4.2. Behavioural Experiments

First Choice

In experiments 1-5 (Table 1), males made first contact equally often with treatment and control stimuli (Table 2), indicating that males were not attracted to any volatile component of the treatment stimuli. When both test stimuli were solvent controls, males made first contact equally often with the stimulus on the right or left side, indicating that there was no directional bias in the bioassay.

Difference between Percentages of Time Spent on Treatment and Control Stimuli

In experiment 1 (Table 1), males spent 40% more time on silk-bearing filter paper than on control paper (Table 2), and 39% more time on silk extract-treated paper than on MeOH-treated paper (Table 2). Males spent 10% more time on paper treated with synthetic MB-MP-S (10 µg) than on MeOH-treated paper (Table 2), and 6.2% more time on the right hand side paper than on the left hand side paper when both papers were MeOH-treated (Table 2). There was no difference between the percentage of time males spent on the treatment filter paper relative to the control filter paper (i.e. the effect sizes) when comparing responses to silk or silk extract (Tukey's HSD, $t = -0.15$, $df = 72$, $P = 1.0$). The time males spent on the filter paper treated with MP-MB-S relative to the control was also not significantly different from the time males spent on the right hand side filter paper relative to the left hand side filter paper when both were treated with methanol alone (Tukey's HSD, $t = -0.39$, $df = 72$, $P = 0.98$). All other pairwise comparisons were significantly different ($P < 0.001$ after adjusting for multiple comparisons within experiment 1, indicated by different letters in Table 2).

In experiments 2-5 (Table 2), naïve males spent 52% more time on paper treated with silk extract than on MeOH-treated paper (Experiment 2), 14% and 16% more time, respectively, on paper treated with synthetic MB-MP-S at 10 µg or 100 µg than on MeOH-treated paper (Experiments 3, 4), and 10% more time on paper treated with silk extract and synthetic MB-MP-S than on paper treated with silk extract alone (Experiment 5).

Courtship

In experiment 1 (Table 1), none of the 25 males tested courted on controls but most responded with silk wrapping to silk and silk extract (96% and 80%, respectively; Table 2). Males also courted in response to synthetic MB-MP-S (10 µg), although at a lower rate (16% in experiment 1, 3% in experiment 3; Table 2). Silk-wrapping response rates (15%) were similar when males were exposed to a higher (100-µg) dose of synthetic MB-MP-S (Table 2). In experiment 5, 89% of the males bioassayed wrapped both test stimuli, the paper impregnated with silk extract alone and the paper impregnated with both silk extract and synthetic MB-MP-S, whereas 11% of males wrapped neither stimulus (Table 2). Two males responded with silk wrapping on the arm

of the T-rod bearing the filter paper treated with the admixture of silk extract and synthetic MB-MP-S even before they made contact with either test stimulus.

2.5. Discussion

We have demonstrated that a courtship-eliciting contact pheromone on the silk of female *L. hesperus* can be extracted with methanol. Male *L. hesperus* prefer synthetic MB-MP-S over controls, but not to the same extent as they prefer silk or silk extract over controls. MB-MP-S occasionally elicits male courtship behaviour, whereas silk and silk extract consistently trigger silk-wrapping. Our results support the conclusion that MB-MP-S is one component of a multi-component pheromone. Below, we discuss the implications of our results for identifying the sex pheromone of *L. hesperus* and other spiders, and highlight the importance of using carefully designed bioassays for testing the various behavioural responses of spiders to chemical signals.

Contact pheromone is extractable with methanol from the silk of virgin female *L. hesperus* and elicits courtship behaviour by males. Males responded just as strongly to methanol extract of silk as they did to silk itself, spending most of their time in contact with silk- or silk extract-treated paper, and wrapping both stimuli with silk. These results indicate that there is a methanol-soluble sex pheromone on the silk that triggers silk-wrapping behaviour. Naïve males spent significantly more time on paper treated with synthetic MB-MP-S (10 or 100 µg) than on MeOH-treated paper but the preference was not as strong as for silk extract. That some males did respond to MB-MP-S with courtship, although less strongly than to extract, suggests that MB-MP-S is a component of the courtship-eliciting sex pheromone. Males also slightly preferred silk extract admixed with synthetic MB-MP-S to silk extract alone, and most males responded with courtship to both stimuli, suggesting that MB-MP-S contributes to the pheromone signal but that other pheromone components in silk extract are required to consistently trigger courtship. Intriguingly, two males began silk-wrapping on the horizontal arm of the T-rod on the side treated with MB-MP-S admixed with silk extract before making contact with the filter paper, indicating that courtship behaviour was induced by one or more volatile pheromone components. In the spider *Agelenopsis aperta* (Agelenidae), the airborne pheromone 8-methyl-2-nonanone, which emanates from the female's body, functions not only as an attractant but also elicits male courtship behaviour (Papke et al. 2001).

The first choice response of male spiders did not indicate attraction to treatment stimuli in any experiment. Because *L. hesperus* males are attracted to females' webs in the field (Kasumovic and Andrade 2004), we had predicted that males would orient first toward silk or silk extract in T-rod experiments. That they did not is not likely due a flight response after being introduced onto the test apparatus. Males typically paused at the intersection of the 'T' and briefly tapped each side of the horizontal arm with their first pair of legs before they walked on, sometimes reversing direction before making contact with a filter paper. We conclude that male *L. hesperus* did not orient toward volatile silk cues in the context of our bioassay. Males may be attracted to females only at a relatively long range (possibly exceeding the 13-cm length of each of the T-rod's horizontal arms), and they may rely primarily on contact pheromone at closer range. Alternatively, wrapping silk around filter papers and thus reducing the surface area exposed to air may have limited dissemination of male-attractant volatile pheromone components. Also, the volatile pheromone components may not be soluble in methanol.

When designing pheromone bioassays for spiders, it is important to consider the context in which the behavioural response being tested normally occurs. Our T-rod bioassay allows male black widows to approach, and engage with, test stimuli from a climbing/hanging position, which is akin to how they would engage with stimuli on a female's web. The silk-wrapping behaviour in this context is easy to see and to interpret as courtship behaviour. Our T-rod bioassay design allowed us to test whether and how male black widows respond to contact chemical stimuli, but it did not reveal any attraction to test stimuli, despite strong field evidence for attraction of male *L. hesperus* to females' webs. In the field, mate-searching males traverse the ground, a context that was not provided in our bioassay. Experiments in the field or in field enclosures remain the only suitable method for testing attraction of male black widows (Kasumovic and Andrade 2004; MacLeod and Andrade 2014).

Of the seven female spider pheromones identified to date, several appear to be single compounds. However, *Pholcus beijingensis* (Pholcidae) females produce a two-component pheromone blend consisting of (*E,E*)-farnesyl acetate and hexadecyl acetate at a 2:1 ratio (Xiao et al. 2009). Even in species where a single compound has been found to have strong pheromonal activity, it is possible that other components contribute to chemical signaling. The contact pheromone of *Tegenaria atrica* (Agelenidae) is a

complex mixture of fatty acids on the silk and cuticle of females, including four compounds, each of which when tested alone elicits courtship by males (Prouvost et al. 1999; Trabalon et al. 2005).

Some spider pheromones appear to have multiple functions. In *A. aperta*, a single compound both attracts males and induces courtship (Papke et al. 2001). In *Linyphia triangulosa*, a single compound elicits courtship, but its breakdown products act as airborne attractants (Schulz and Toft 1993; Schulz 2013). However, different pheromone components or combinations of components may have distinct functions. In studies of spider pheromones, commonly only one behavioural response, or type of bioassay for pheromonal activity, is reported. For example, the contact pheromone of *L. hasselti* elicits strong overall activity of males, but specific courtship behaviours were not recorded, and the pheromone was not tested as an attractant (Jerhot et al. 2010). Perhaps the *L. hasselti* contact pheromone primarily stimulates male activity on the web, but other components are necessary to elicit specific courtship behaviours like silk-wrapping, as we found for MB-MP-S in our study with *L. hesperus*. In the genus *Latrodectus*, pheromones on the silk of females not only attract males and elicit courtship behaviour, but also provide information about the age, reproductive status, and body condition of the female (Andrade and Kasumovic 2005; Stoltz et al. 2007; MacLeod and Andrade 2014). Possibly, each of several components, or complex blends thereof, have distinct functions or carry specific information about the signaler. As more spider pheromones are identified, and tested for a variety of functions, we will discover whether multi-component pheromones and/or multiple pheromones with distinct functions are common.

One challenge of studying the pheromones of spiders is that numerous and varied compounds are present on the silk and cuticle (Schulz 2013). Behavioural responses of males may be subtle or occur only under certain conditions, and thus may not always be the best indicators of pheromonal activity. In studies of insect semiochemicals, samples are routinely subjected to gas chromatographic-electroantennographic detection (GC-EAD) analyses (Arn et al. 1975). This method allows researchers to determine the chemicals in the gas chromatogram that elicit responses from the insect antenna, thus limiting the number of compounds to be tested for pheromonal activity to only those that the insect can sense. Analogous

electrophysiological screening for spider pheromones would be highly advantageous, but spider olfaction and contact chemoreception are still poorly understood, and only few examples are reported in the literature. In *Cupiennius salei* (Ctenidae), contact chemoreceptors on the dorsal surface of the male's pedipalps detect the female sex pheromone dimethyl citrate (Tichy et al. 2001). Similarly, results of 'electrotarsograms' with the tarsi of female *Pholcus beijingensis* implied the presence of receptors that detected the volatile male aphrodisiac pheromone (*Z*)-9-tricosene (Xiao et al. 2010). 'Electrolegograms' have already been developed for whip spiders (Amblypigi; Hebets and Chapman 2000). Analogous work focused on spider neurophysiology should supplement and corroborate the results of behavioural bioassays of spider pheromones, and lead to a more nuanced understanding of spider chemical signaling.

Acknowledgements

We thank the Tsawout First Nation for permission to do field work on their lands, the Capital Regional District for granting permits to collect spiders from Island View Beach; Maydianne Andrade, Bernard Roitberg, Tanya Stemberger and Samantha Vibert for valuable comments that improved the study and/or the manuscript; Huimin Zhai for comments on the chemistry section; and Susan Chen and Elaine Wu for assistance with spider rearing and maintenance. Funding was provided by a Natural Sciences and Engineering Research Council of Canada (NSERC) – Discovery Grant and an NSERC-Industrial Research Chair to G.G., with Contech Enterprises Inc. and Global Forest Science as industrial sponsors.

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Table 2.1 Details of T-rod choice experiments 1 to 5, testing the behavioural responses of male *Latrodectus hesperus* to various stimuli

Exp.	<i>n</i>	Treatment ^a	Control ^a	Max. time ^b
1 ^c	25	Silk (1 FW ^d)	Blank paper	30 min
		Silk extract (1 FWE ^e)	Methanol	30 min
		Synthetic MB-MP-S (10 µg)	Methanol	30 min
		Methanol (right side)	Methanol (left side)	30 min
2	39	Silk extract (1 FWE ^e)	Methanol	15 min
3	39	Synthetic MB-MP-S (10 µg)	Methanol	15 min
4	20	Synthetic MB-MP-S (100 µg)	Methanol	15 min
5	36	Silk extract (0.5 FWE) plus synthetic MB-MP-S ^f (10 µg)	Silk extract (0.5 FWE)	30 min

^aSilk extract, synthetic MB-MP-S or corresponding methanol control stimuli were each applied to filter paper at 40-µL aliquots;

^bTrials were terminated when the maximum time had elapsed or the male had dropped down to the substrate and walked away, whichever came first;

^cEach of 25 males was tested with all four treatments in random order, with one test per day on consecutive days; males in all other experiments were tested only once;

^dFW = female's web;

^eFWE = female web equivalent;

^fMB-MP-S = *N*-3-methylbutyryl-*O*-methylpropanoyl-*L*-serine methyl ester

Table 2.2 Responses of *Latrodectus hesperus* males in behavioural bioassays (see Table 1 and methods for details of treatment and control stimuli) on T-rods (Fig. 1). For each experiment, the percentage of males that made contact with the filter paper on the treatment side of the T-rod first and the percentage of males that engaged in silk-wrapping behaviour in response to test stimuli are given with 95% Confidence Intervals (CIs) calculated using the Agresti-Coull method. For each experiment, the mean of the differences between the percentage of time each male spent on the treatment filter paper compared to the control filter paper are given with 95% CIs. Different capital letters indicate means that differ significantly within experiment 1 based on an ANOVA followed by Tukey's HSD (experiment-wise $P < 0.05$). Also shown are the results of individual t -tests for each experiment, indicating whether males spent a significantly greater percentage of time on the treatment filter paper than the control filter paper

Exp.	n	Treatment (Control)	Chose treatment side first [% of males] (95%CI)	Time spent on treatment minus time on control [% of total time] (95% CI)	t	df	P	Silk-wrapped [% of males] (95% CI)
1	25 ^a	Silk [1 FWE ^c] (Blank paper)	52 (33,70)	40 (26,55) A	5.85	24	<0.001	96 (78,100) ^f
		Silk extract [1 FWE ^d] (Methanol)	40 (23,59)	39 (24,54) A	5.32	24	<0.001	80 (60,92) ^f
		MB-MP-S ^e [10 µg] (Methanol)	52 (33,70)	10 (0,19) B	1.99	24	0.058	16 (6,35) ^f
		Methanol right side (Methanol left side)	56 (37,73)	6 (-1,14) B	1.72	24	0.099	0 (0,16) ^g
2	39 ^b	Silk extract [1 FWE] (Methanol)	41 (27,57)	52 (38,65)	7.87	38	<0.001	92 (79,98) ^f
3	39 ^b	MB-MP-S ^e [10 µg] (Methanol)	54 (39,68)	14 (5,24)	2.99	38	0.005	3 (0,14) ^f
4	20 ^b	MB-MP-S ^e [100 µg] (Methanol)	55 (34,74)	16 (7,24)	3.92	19	0.001	15 (4,37) ^f
5	36 ^b	Silk extract [0.5 FWE ^d] + MB-MP-S ^e [10 µg] (Silk extract [0.5 FWE ^d])	58 (40,70)	10 (2,18)	2.46	35	0.019	89 (74,96) ^h

^aEach of 25 males was used in all four tests, in random order;

^bEach male was used in only one test, and had not previously been exposed to female silk/pheromones;

^cFW = female's web;

^dFWE = female web equivalent;

^eMB-MP-S = *N*-3-methylbutyryl-*O*-methylpropanoyl-*L*-serine methyl ester;

^fMales that silk-wrapped on the treatment (no males wrapped controls);

^gMales that silk-wrapped on either side;

^hMales that silk-wrapped on both sides (all other males did not wrap either side)

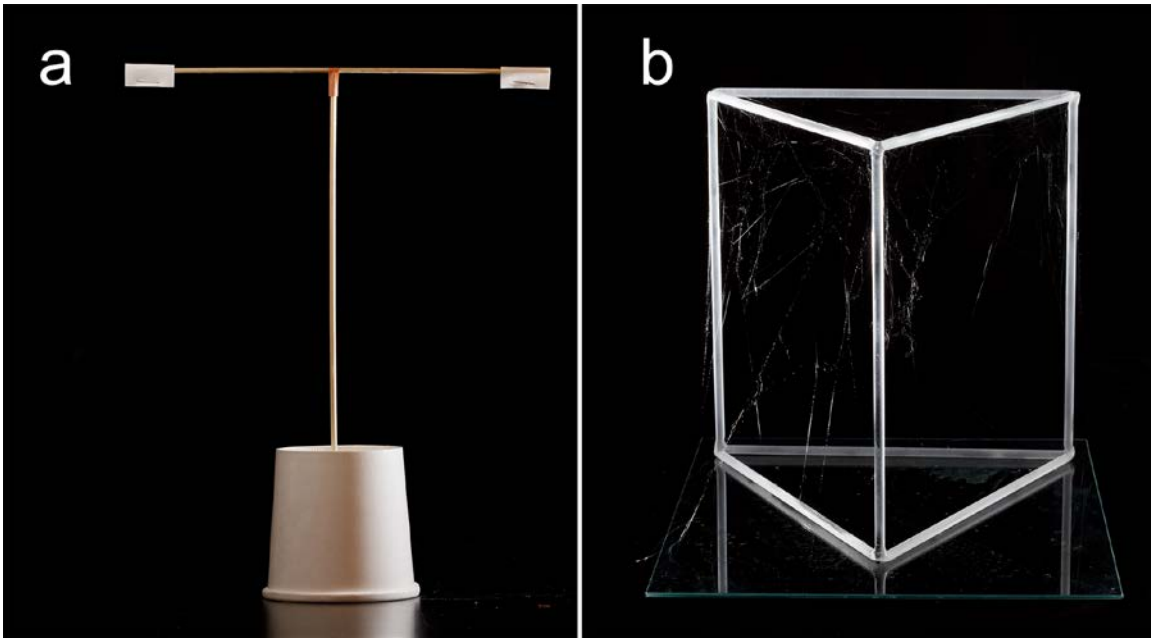


Figure 2.1 (a) T-rod used for testing behavioural responses of *Latrodectus hesperus* males to test stimuli applied to filter paper envelopes on each end of the horizontal rod; (b) Glass frame with web of a female *L. hesperus*; after 72 h of web building, we collected the silk and extracted it with methanol

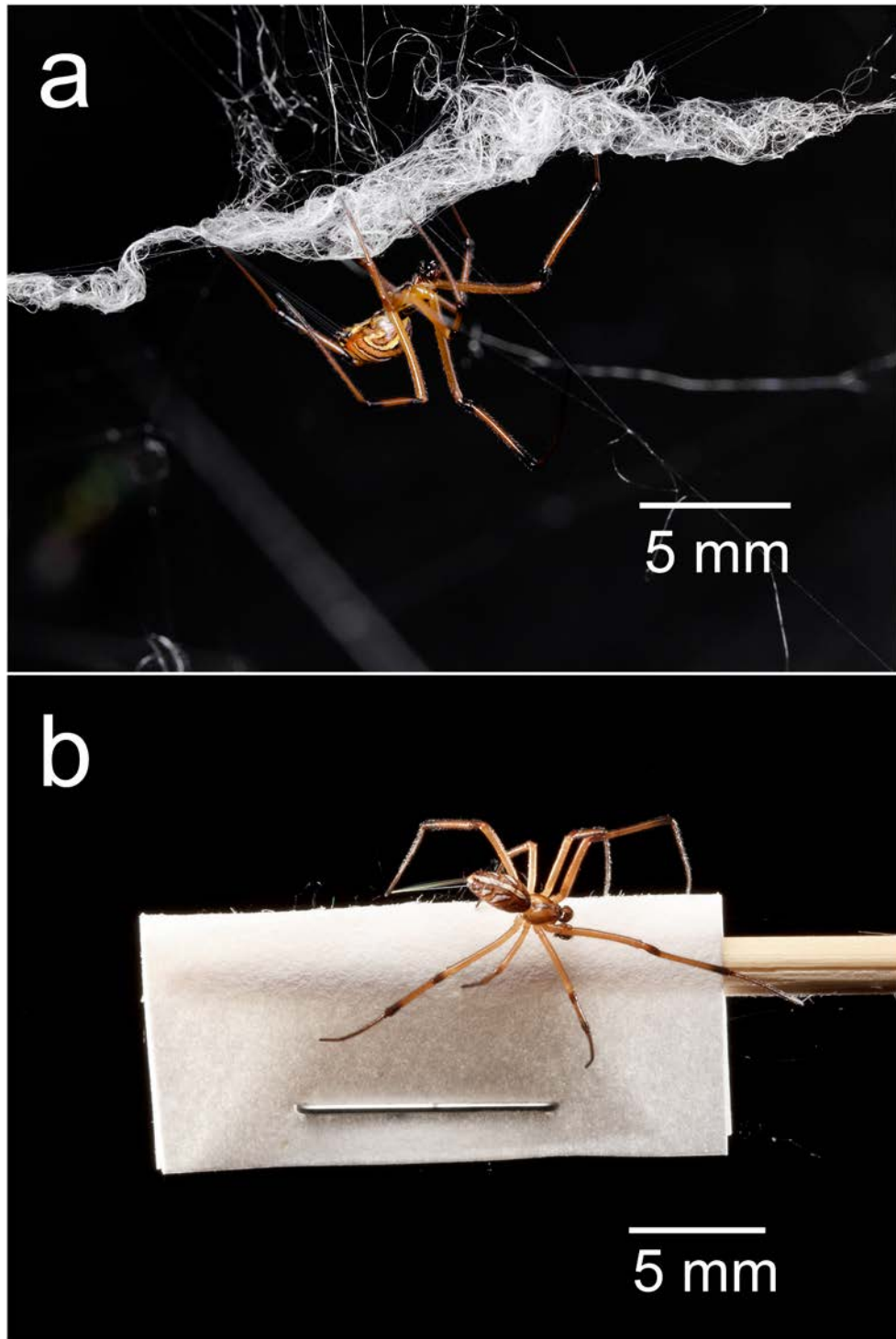


Figure 2.1 (a) Male *Latrodectus hesperus* silk-wrapping as part of his courtship behavior on a female's web; (b) male *L. hesperus* silk-wrapping on a filter paper treated with methanol silk extract. Note in (a) the male's raised abdomen (relative to his carapace), and in (a) and (b) the silk being pulled from his spinnerets with his last pair of legs

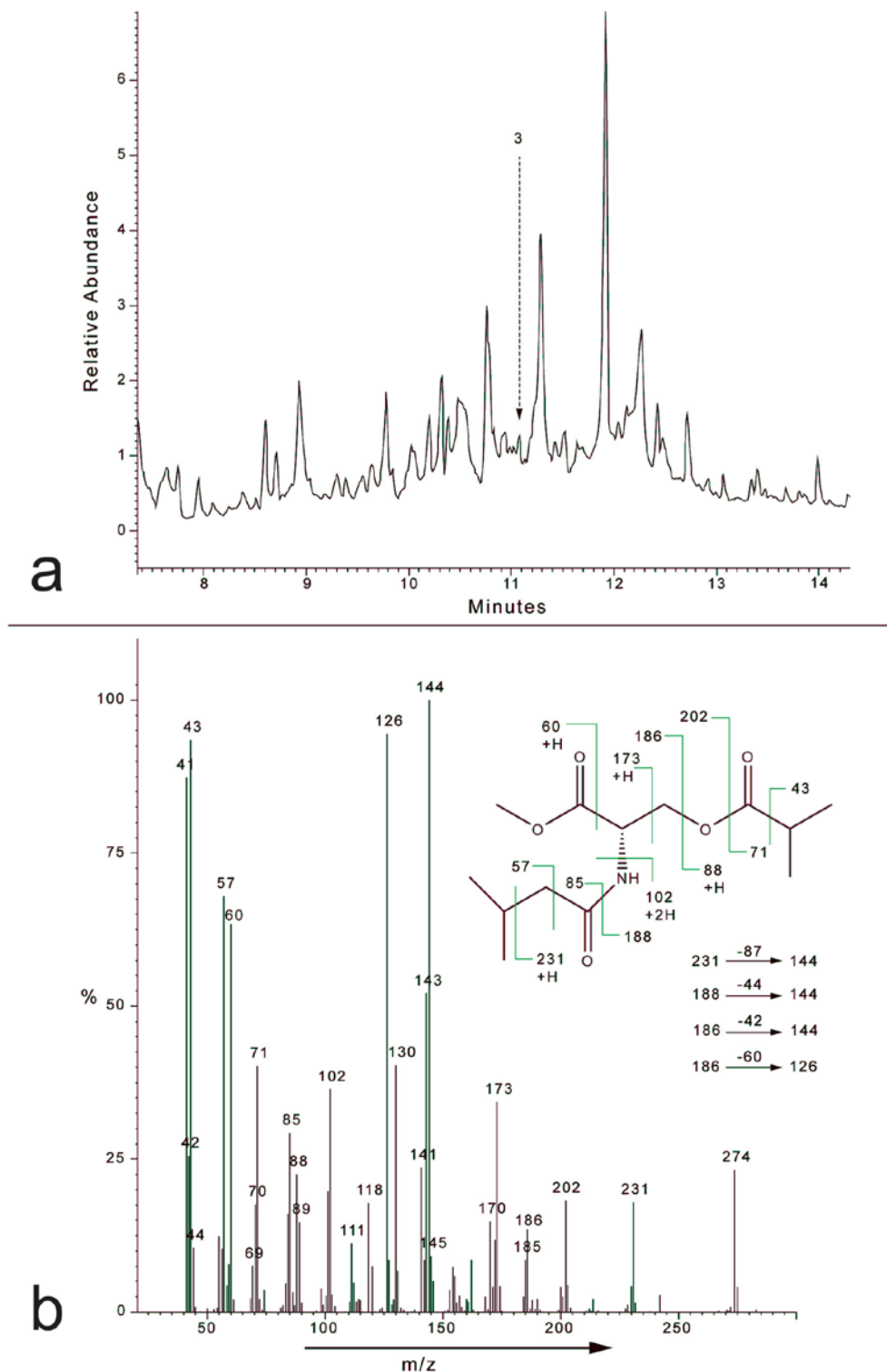


Figure 2.2 (a) Total ion chromatogram (Saturn 2000 Ion Trap GC-MS) of a methanol extract of 21 webs of virgin *Latrodectus hesperus* females; A denotes the candidate pheromone component *N*-3-methylbutyryl-*O*-methylpropanoyl-*L*-serine methyl ester (MB-MP-S); (b) mass spectrum and fragmentation pattern of A

Chapter 3.

Web reduction by male black widows makes pheromone-emitting females' webs less attractive to rivals

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This chapter has been submitted to Behavioral Ecology.

3.1. Abstract

Male adaptations that limit sperm competition include guarding females, applying mating plugs, and chemically reducing the attractiveness or receptivity of females. In many web-building spider species, females attract males with silk-borne volatile pheromones. In widow spiders (*Latrodectus*, 30 species), the courting male often engages in web reduction behavior during which he excises and bundles sections of the female's web and wraps them with his own silk. Hypotheses for the function of this widespread behavior have never been tested in the field. In a dense population of western widows (*L. hesperus*), we ran mate-attraction experiments to test the hypothesis that web reduction and/or male silk addition decrease web attractiveness to potential rivals. Webs reduced by males attracted three times fewer males than intact webs; webs with a similar proportion of silk experimentally removed attracted as many males as intact webs. However, the experimental addition of male silk did not affect the attractiveness of intact webs. We conclude that web reduction in black widows limits male-male competition by reducing attraction of rival males to females' webs. This effect is likely mediated through strategic silk excision by courting males, possibly in combination with the male's silk forming a physical barrier to pheromone emission.

3.2. Introduction

In polyandrous animals males have diverse defensive adaptations that reduce sperm competition (Birkhead and Møller 1998), including preventing or impeding female re-mating (Parker 1970) by mate-guarding, applying a mating plug, or chemically altering female attractiveness or receptivity. These adaptations may conflict with the female's interests if they have costly side effects or if the female benefits from re-mating (Stockley 1997; Arnqvist and Rowe 2005). Conversely, females sometimes benefit from such adaptations; for example, *Pieris* (Pieridae) butterfly males limit costly harassment of mated females by transferring an anti-aphrodisiac pheromone during copulation (Andersson et al. 2000). Studying adaptations that limit sperm competition can provide insights as to how potentially conflicting male and female sexual strategies shape mating systems.

Male spiders use various tactics that help limit or prevent sperm competition, including mate guarding and applying mating plugs (Elgar 1998). In many web-building spider species, males are attracted to volatile pheromones released from the bodies or silk of females, and use chemical cues to discriminate between virgin and mated females (Gaskett 2007; Schulz 2013; Uhl 2013). Whether post-copulatory changes in female attractiveness are under female or male control is not yet clear. Regardless, males should be able to avoid sperm competition effectively if females become unattractive to rival males immediately after mating. However, male spiders may face one or more of the following challenges: (i) fierce competition for access to females, (ii) risk of cannibalism by aggressive females, or (iii) lengthy courtship and mating. One solution may be to reduce female attractiveness during courtship. For example, courting males of the Sierra Dome spider *Neriene litigiosa* (Linyphiidae) excise large areas of the pheromone-laden webs of virgin females and pack the silk into dense bundles. Laboratory studies have shown that such reduced webs are less attractive than intact webs to other males (Watson 1986). Mated females rebuild their webs without attractive pheromone; consequently, a male that performs web reduction decreases the risk of competitors arriving at a female's web both during courtship and after copulation (Watson 1986). Web reduction in *N. litigiosa* may thus be considered a defensive adaptation against sperm competition.

The mechanism underlying decreased attractiveness of reduced webs has never been studied, but the compaction of the web into tight bundles is thought to limit release of the female's pheromone by decreasing the exposed surface area of silk (Watson 1986). One non-exclusive alternative is that males add anti-aphrodisiacs to the female's silk during web reduction (Willey Robertson and Adler 1994), and that these chemical cues repel rivals. The ability to detect and avoid webs of females that are currently occupied, or have been previously occupied, by other males should be favored in species with strong first male sperm precedence. Studies on spider chemical communication have focused on female pheromones (Gaskett 2007; Schulz 2013; Trabalon 2013; Uhl 2013) and although male spiders also emit pheromones from their bodies or silk, they have received little attention. For example, courting *Agelenopsis aperta* (Agelenidae) males emit a volatile pheromone that induces female catalepsis (Becker et al. 2005); pheromones emitted by *Evarcha culicivora* (Salticidae) males and their silken draglines facilitate mate recognition and elicit courtship behavior by females (Cross and Jackson 2013). Web reduction behavior is common in sheet weaver (Linyphiidae) (van Helsdingen 1965; Rovner 1968; Stålhandske and Gunnarsson 1996) and tangle-web (Theridiidae) spiders (Locket 1927; Breene and Sweet 1985; Whitehouse and Jackson 1994). During web reduction, a male dismantles sections of a female's web, bundles and wraps the sections with his own silk, and then often leaves the bundles hanging near the female's retreat (Anava and Lubin 1993). This may be a means of disseminating sex pheromone during intersexual communication (Anava and Lubin 1993), or chemicals on the male's silk may render the female's pheromone unattractive (Willey Robertson and Adler 1994). Males of the orb weaver *Nephila senegalensis* (Nephilidae) avoid webs that have previously been visited by another male based on cues associated with his silk draglines (Schneider et al. 2011). Similarly, the reduced attractiveness of reduced webs may be due to males avoiding silk deposited by courting males because this silk emits a male sex pheromone, anti-aphrodisiac pheromone, or both.

Web reduction is a common element of long, normally nocturnal pre-copulatory displays in black widows (*Latrodectus* spp., Theridiidae) (Breene and Sweet 1985; Forster 1995; Segoli et al. 2008; Harari et al. 2009). *Latrodectus* males typically remove approximately half of a female's web during web reduction, wrapping the bundled silk extensively with their own (Anava and Lubin 1993; Scott et al. 2012) (Figure 1). Male

western black widows, *L. hesperus*, produce a silk-borne contact pheromone that elicits female courtship behavior (Ross and Smith 1979), and immature males of a congener adjust their development based on volatile chemical cues from conspecific males (Kasumovic and Andrade 2006). Whether web reduction in black widows or other theridiid spiders curtails male-male competition or serves in intersexual communication has never been studied. Here we investigate the function of web reduction in *L. hesperus*, where both direct competition for mating opportunities and sperm competition have likely shaped male reproductive strategies. Male *L. hesperus* prefer virgin to mated females based on long-range pheromone cues (MacLeod and Andrade 2014), and some males guard sub-adult females (Ross and Smith 1979). Males will benefit from adaptations that improve their ability to monopolize females because polyandry occurs in the field and copulatory plugs (the broken-off tips of male intromittent organs) are often ineffective at preventing subsequent mates from siring offspring (MacLeod 2013).

Here, in studies of a dense field population of *L. hesperus* (Salomon et al. 2010) with the potential for intense male-male competition, we show that male-performed web reduction reduces the attractiveness of female webs to rival males. We also tested two hypotheses for the mechanism(s) underlying this effect. First, we asked whether a decreased silk surface area alone is sufficient to cause decreased web attractiveness by experimentally excising 50% of the female's web silk (comparable to the average web reduction by males). Second, we asked whether males avoid the silk deposited by a web-reducing male by examining attraction of males to intact webs to which we had experimentally added sections of female web bundled and wrapped with male silk.

3.3. Materials and Methods

3.3.1. Study area and animals

Our study site was a 20 × 400 m area of coastal sand dunes above the high-tide line at Island View Beach, on the Saanich peninsula of Vancouver Island, BC, Canada (48° 34' N, 123° 22' W; elevation 3-5 m). At this site, *L. hesperus* females build their webs under driftwood logs and other woody debris; 2-3 sub-adult or adult females were found per m² of suitable habitat during surveys each September from 2002–2005 (Salomon et al. 2010). On 26 May 2013, we collected 50 mated female *L. hesperus*. We

reared their offspring to maturity in the laboratory on a diet of house crickets (*Acheta domestica*) and blow flies (*Lucilia sericata* and *Phormia regina*). When not being used for experiments, we kept all spiders individually in Petri dishes (150 × 25 mm for females; 100 × 25 mm for males) at 20–25 °C on a reverse 12:12 h (light:dark) photo regime. Once spiders had reached maturity, we fed females at least one large cricket or four blow flies per week. We provided adult males with a few drops of water weekly but did not feed them because they normally do not eat after their final mount. For web-building and web reduction, we used virgin females less than six months post-maturity and males less than three weeks post-maturity, respectively.

3.3.2. Mate-attraction assays in the field

At our field site, we examined attraction of males to screen cages containing webs from which females had been removed.

(i) General experimental methods

We built cages (26 cm diameter × 20 cm tall; see Figure 2a) from aluminum window screening (18 × 16 mesh; New York Wire, Hanover, Pennsylvania, USA) and plastic plates (26 × 26 cm; Solo Squared™, Solo Cup Company, Lake Forest, Illinois, USA). We surrounded each cage with 10-cm wide strips of waxed cardboard coated with a thin layer of adhesive insect trap coating (Tangle-trap, Contech Enterprises, Victoria, BC, Canada). We placed females in separate wire mesh cages seven days before the field experiment, not feeding spiders until after the experiment so that no prey odor would be present in cages.

At our field site we established a 380-m transect adjacent to a walking path and placed trap replicates approximately every 20 m along the transect (Figure 2b). We selected locations that were ~1 m away from at least one driftwood log, which is suitable black widow habitat (Salomon et al. 2010). We placed traps on the beach at sunset, and checked them every 3 h for 24 h. We counted and collected all males that we found on the sticky strips surrounding each cage, and occasionally on the cages themselves, and preserved them in 95% ethanol. The few males not stuck to the adhesive strips likely reached cages via silk lines from nearby vegetation.

Our experiment was designed to ensure that any males we captured responded primarily to volatile chemical cues associated with female or male silk inside cages. Widow spiders are nocturnal and have poor vision (Foelix 2011), therefore visual cues are not likely to have affected the males' responses (also see MacLeod & Andrade, 2014).

(ii) Experiment 1: Effect of natural and experimental web reduction on web attractiveness

Less than 3 h before starting the field experiment, we removed females from their webs and prepared 20 replicates of each of the following four treatments: cages containing (1) intact webs (no manipulation), (2) male-reduced webs (on average ~50% of web silk bundled and wrapped with male silk; see below), (3) mechanically reduced webs (~50% of web silk excised), and (4) no webs (control). We included no-web control cages to confirm that captures of males on adhesive strips around cages resulted from attraction of males to female silk rather than random mate searching.

We used a randomized complete block experimental design, with each treatment appearing once in each block. We randomly assigned treatments to the four vertices of a randomly oriented 1 × 1 m square. To minimize variation in web attractiveness within each block, we used webs spun by females that matured within 14 days of one another and whose body masses (mean: 343.6 (SD 56.8) mg) differed as little as possible. The mean largest mass difference among the three females within each block was 39.3 (SD 32.5) mg.

To obtain male-reduced webs, we introduced a male onto each of 20 empty webs at the farthest possible location from the female's retreat and allowed him to engage in courtship for 1 h. Males readily court on empty webs of virgin females (Ross and Smith 1979; Vibert et al. 2014). Males usually begin web reduction within the first 15 min of courtship, and continue to engage in this behavior for up to 1 h (C. Scott; unpublished data). If after 15 min a male had not moved at all, or had not begun web reduction behavior, we replaced him with a new male who was allowed to court for 1 h. The extent to which males reduced webs was variable, ranging from no visible web reduction to most of the web being reduced to a dense rope or ball (Figure 1). Based on visual inspection of reduced webs, we estimate that on average 50% of the web area was removed and wrapped with male silk ($\leq 25\%$ reduced: 5 webs; $\sim 50\%$ reduced: 9 webs;

≥75% reduced: 6 webs). We removed males from reduced webs immediately before transporting cages to trap locations on the beach.

We prepared mechanically-reduced webs by cutting webs in half with scissors along a straight line extending outward from the female's retreat. The silk threads of the web are usually most concentrated around the female's retreat, and the web becomes less dense with increasing distance from the retreat. We randomly selected the right or left half of the web for silk removal. Because tangle-webs have a three-dimensional structure, the remaining half of the web remained intact, with minimal changes in structure due to reduced tension in some of the threads. We chose to remove approximately 50% of the web because this is the typical extent of web reduction during *Latrodectus* courtship (Anava and Lubin 1993; Scott et al. 2012).

(iii) Experiment 2: Effect of male silk on web attractiveness

To test whether the male's silk on females' webs affects the attraction of males, we conducted a second experiment three weeks later, using the same design as above except that our 20 replicates included a paired-treatment design with cages containing either (1) intact webs (no manipulation) or (2) intact webs with male silk added (see below). To minimize within-replicate variation in web attractiveness, we chose two females for each replicate that had matured within 14 days of one another and whose masses (331.5 (SD 63.4) mg) differed as little as possible (mean mass difference: 16.6 (SD 14.6) mg). We used a third group of 30 females (mean mass: 350.45 (SD 54.0) mg) to obtain male silk produced in the process of web reduction. Less than 3 h before the experiment, we removed all females from their webs, introduced a male onto each of the 30 empty webs and allowed each male to court for 1 h. For collection of male silk, we then chose 20 visibly reduced webs with an obvious ball or rope of female web wrapped in male silk (Figure 1). We used scissors to excise such male silk-wrapped web balls or ropes, and forceps to place them in the center of a web randomly selected from each pair of intact treatment webs. Thus, webs with male silk added comprised an entire female's web plus an unknown quantity of a second female's web wrapped with male silk. In each replicate, we randomly assigned the intact web and the intact web with male silk added to one of two trap locations, with 1 m spacing between paired traps and approximately 20 m spacing between trap pairs (Figure 2).

3.3.3. Statistical analyses

For each experiment we used a generalized linear model with a negative binomial distribution and log link to assess the effects of treatments and blocks on the number of male spiders captured over 24 h. For experiment 1 we ran post-hoc tests to determine whether there were differences between treatments and adjusted P -values for multiple comparisons using the Tukey method for four means (raw data provided in supplementary file S1). For experiment 2 we excluded one block that did not capture any spiders from the analysis (raw data provided in supplementary file S2). We used R 3.0.2 for all data analyses (R-Core-Team 2013), the function `glm.nb` in the MASS package (Venables and Ripley 2002) to run the models, and the `lsmeans` package (Lenth 2014) for calculations of least squared means and 95% confidence intervals, and post-hoc comparisons between means.

3.4. Results

3.4.1. Experiment 1: Effect of natural and experimental web reduction on web attractiveness

We captured 230 *L. hesperus* males over 24 h across all traps. Most (155) of these males responded during the first 6 h of the experiment, between 2100 and 0300 h (Table 1). Boxplots summarizing the data for each of the four treatments are shown in Figure 3.

The negative binomial GLM indicated that there was a significant effect of treatments ($F_{3,57} = 10.71$; $P < 0.001$), but not blocks ($F_{19,57} = 0.72$; $P = 0.79$), on the number of male spiders captured. Male-reduced webs attracted fewer males than did either intact or mechanically reduced webs. Intact webs attracted 3.07 (SE 0.84) times as many males as did male-reduced webs ($t_{57} = 4.09$, $P < 0.001$), and mechanically reduced webs attracted 2.31 (SE 0.65) times as many males as did male-reduced webs ($t_{57} = 2.99$, $P = 0.02$). In contrast, the attractiveness of intact and mechanically reduced webs did not differ significantly ($t_{57} = 1.173$, $P = 0.65$). All three web-containing treatments captured more males than did the no-web controls ($P < 0.01$ for all three comparisons).

3.4.2. Experiment 2: Effect of male silk on web attractiveness

In experiment 2, we captured 461 *L. hesperus* males over 24 h across all traps. Most (268) of these males responded during the first 6 h of the experiment, between 2030 and 0230 h (Table 2). Intact webs and webs with male silk added captured 242 and 219 male spiders, respectively (boxplots are shown in Figure 4). There was no effect of treatments ($F_{1,18} = 0.11$; $P = 0.74$), or blocks ($F_{18,18} = 0.49$; $P = 0.93$), on the number of male spiders captured.

3.5. Discussion

Our field experiments demonstrate that web reduction by male *L. hesperus* results in a three-fold decrease in web visits by rival males, thereby limiting male-male competition. Below, we discuss the fitness consequences of web reduction for both sexes, arguing that the benefits of web reduction for females may outweigh the costs, even though conflict may arise over web damage. Web reduction with male silk deposition decreased web visits by males whereas experimental removal of half of the web did not. However, the addition of male silk to intact webs had no effect on their attractiveness. Based on these results, we conclude that strategic excision of pheromone-laden web sections and male silk wrapped around excised sections acting as a barrier to pheromone emission are the most likely mechanisms by which web reduction decreases web attractiveness.

3.5.1. Consequences of web reduction

Our data provide evidence of strong male-male competition in a field population of western black widow spiders. We captured 224 *L. hesperus* males arriving at 60 webs during our first 24-h experiment, and 461 males at 40 webs during our second 24-h experiment. The median number of males arriving at a single female's intact web within the first 6 h was 3.8 (range: 0-13) and 7.5 (range 0-21) in the first and second experiment, respectively. Because courtship and copulation in *L. hesperus* typically last several hours (Scott et al. 2012; MacLeod 2013), the arrival of multiple males at a female's web within hours of one another poses a substantial risk that the courtship or copulation of an early arriving male will be interrupted by rivals. In contrast, the median

number of males arriving within the first 6 h at male-reduced webs was 0 (range: 0-5), showing that males engaging in web reduction immediately upon entering a virgin female's web could limit the number of rival males arriving at the web, and thus greatly decrease direct competition over access to the female.

Latrodectus males can prevent sperm competition by depositing mating plugs in the female's reproductive tract (Berendonck and Greven 2000; Snow et al. 2006; MacLeod 2013). Although this tactic contributes to a general pattern of first male sperm precedence in *L. hesperus*, it often fails (MacLeod 2013). Consequently, a male that prevents rivals from arriving on the web of a female both during courtship and after mating will significantly reduce his probability of facing sperm competition. Female *L. hasselti* cease production of contact sex pheromone immediately after mating (Stoltz et al. 2007; Jerhot et al. 2010), and *L. hesperus* males discriminate against mated females in favor of virgin females based on volatile pheromone cues alone (MacLeod and Andrade 2014). Thus, the effect of web reduction is likely to be long lasting because mated females will rebuild their webs without attractive pheromones.

Web reduction may be a particularly efficient means of guarding against female re-mating because it does not require the physical presence of a male on a female's web. Mate searching is generally risky for *Latrodectus* males (Andrade 2003; Segoli et al. 2006), severely limiting opportunities for polygyny, and favoring investment in a single mating. However, *L. hesperus* males can mate multiple times, even after genital damage (MacLeod 2013). Moreover, the dense population of female *L. hesperus* at our field site (Salomon et al. 2010) makes it likely that a mated male can readily locate a second mate. Indeed, we have often found the webs of two or more adult females in close proximity under the same log, which would allow a male that mates one female to go on to visit additional females with minimal energetic cost and predation risk. In this system, males may have the potential to increase their fitness by both mating with more than one female and remotely guarding those females via web reduction.

Not all *L. hesperus* males engage in web reduction during courtship (Scott et al. 2012) and there is considerable variation in the extent to which a web is reduced (this study). Silk is costly to produce (Craig 2003) and is likely a limiting resource for *Latrodectus* males who do not forage as adults (Foelix 2011). Web reduction may have two distinct functions: (1) limiting the arrival of competitors (Watson 1986) and (2)

signaling to the female (Ross and Smith 1979; Anava and Lubin 1993). We speculate that the former may depend on the amount of web reduced, whereas the latter may depend on the amount of male silk added. Males might then adjust their investment into destroying a web and depositing their own silk according to the competitive environment, their own condition, and the female's receptivity. If *L. hesperus* males assess their competitive environment using chemical cues (Kasumovic and Andrade 2006), and are capable of parasitizing the courtship effort of other males (Stoltz and Andrade 2010), as in *L. hasselti*, this could explain why males did not invariably avoid reduced webs in the field. Silk draglines from captured males were visible around the cages in our experiments, and could have provided cues that allowed males to assess the local level of competition for females. Males that are not likely to win fights for access to females because they are small, or that cannot afford to invest in silk deposition or web reduction because they are in poor condition, may benefit from adopting alternative mating tactics (Stoltz et al. 2008). Males that locate and enter webs that are in the process of being reduced may be able to sneak copulations with the female while the other male invests in lengthy courtship behavior (Stoltz et al. 2008).

That females tolerate web reduction by males is somewhat puzzling because web damage appears to conflict with female interests through lost opportunities for prey capture, energy and silk required for web repair, and increased predation risk (Schneider and Lubin 1998). Furthermore, fewer web visits by male suitors as a result of web reduction may be costly if females benefit from mating with multiple males (Watson 1998). Conversely, if repeated courtship and re-mating are costly for females (Arnqvist and Rowe 2005), mated females should benefit from changes that limit the arrivals of additional males. Silk of virgin females remains attractive and continues to elicit courtship behavior in males for several days, even in the female's absence (Ross and Smith 1979; Andrade and Kasumovic 2005). Web reduction may benefit females by allowing them to cease attracting males immediately upon mating, and then to remain unattractive by rebuilding their webs without pheromones. Web reduction may not necessarily limit opportunities for mate choice or polyandry, however, if several males arrive at a web within hours of one another, as found at our study site. Furthermore, the effect of web reduction on female attractiveness need not be permanent if females can re-advertise their receptivity several months after mating, as do *L. hasselti* females (Perampaladas et al. 2008).

3.5.2. Proximate mechanisms – how does web reduction alter web attractiveness?

Our data do not support the hypothesis that males avoid silk deposited by another male during web reduction. There was no difference in the number of males arriving at intact webs with or without added male silk-wrapped web bundles. We may not have seen an effect in this study because we tested male silk in the presence of female sex pheromone emanating not only from an entire intact web but possibly also from the added male silk-wrapped bundle of female silk. The dragline silk that males deposit all over the web during courtship may also function in limiting web attractiveness but this silk was absent in our experiment. Conceivably, males detect and avoid male silk on a female's web only when a portion of her web has been destroyed and wrapped with male silk, perhaps limiting pheromone emission and resulting in a distinct ratio of male to female silk cues. With strong competition for access to females at our field site, and first male sperm precedence in *L. hesperus* (MacLeod 2013), the best strategy for males might be to avoid webs that have been largely reduced (high ratio of male to female silk cues) and therefore present a risk that mating is in progress or already complete, but enter webs that have just begun to be reduced (low ratio of male to female silk), where courtship has only recently begun. The possibility that mate-seeking male spiders respond to chemical cues from male silk deposited during web reduction warrants further study.

Our results in experiment 1 suggest that web reduction may not limit a female's attractiveness simply by decreasing the silk surface area available for pheromone release. Webs with half of the silk experimentally excised were visited by as many males as were intact webs, indicating that lowering pheromone release by 50% on average has no effect. This is expected, given that in the spider *Agelenopsis aperta* the amount of female sex pheromone must be lowered more than 100-fold to reduce male responses by half (Papke et al. 2001). In our study the webs from which we excised 50% of the silk attracted more than twice as many males as did webs from which males had cut, bundled, and wrapped 50% of the silk on average. Even if pheromone is not evenly distributed throughout the web, mechanically reduced webs had on average 50% of the female's sex pheromone removed entirely, while male-reduced webs retained the full complement of female pheromones. In his study of web reduction by *Neriene litigiosa*, Watson (Watson 1986) suggested that compaction of a female's silk into a tight mass

limits pheromone release by reducing the exposed silk surface area, and did not notice male silk addition. The web bundles formed by *L. hesperus* males during web reduction are often rather loose (see Figure 1), and thus a decrease in silk surface area exposed to air alone does not seem likely to explain the large decrease in attractiveness of male-reduced webs. We suggest that strategic excision of pheromone-rich sections of the web, coupled with a barrier to pheromone release formed by the male's added silk, is the most likely mechanism by which web reduction decreases web attractiveness in widow spiders. It is also plausible that chemicals associated with male silk bind to, alter, or neutralize pheromone on the female's silk. The likelihood of these speculative mechanisms is unclear because we know little about how spider pheromones are incorporated into the silk or how they are sensed (Uhl 2013).

3.6. Conclusion

Through web reduction, black widow males appear to 'mute' the attractive signals produced by females, perhaps also altering them through addition of silk-borne chemicals. Male manipulation of female signals by chemical deposition is common in insects, and may occur by direct contact with the female, or by transfer with the ejaculate or a mating plug (Thomas 2011). Web reduction is unusual in that it takes place early during courtship, before the male makes contact with the female; male alteration of female attractiveness or receptivity prior to mating is rarely reported for insects (but see Ablard, Schaefer, & Gries, 2013). Web reduction appears to be a means by which a male can monopolize a female during a long courtship display and continue to remotely guard her after copulation, complementing other defenses against sperm competition including mating plugs. Our results provide the basis for comparative studies of the function of web reduction in other spider species. Among the widow spiders alone, there is extensive variation among mating systems and population densities. Studying this behavior and the mechanisms by which it functions in mate monopolization and intra- or inter-sexual communication will provide new insights into how mating tactics, sexual selection, and conflict shape mating systems.

Funding

This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) – Discovery Grant and by an NSERC – Industrial Research Chair to GG, with Contech Enterprises Inc. and Global Forest Science as industrial sponsors.

Acknowledgements

We thank the Tsawout First Nation for permission to do field work on their lands, and the Capital Regional District for granting permits to collect spiders from Island View Beach. We thank M. Andrade, R. Bennett, G. Blackburn, B. Roitberg, and S. Vibert for valuable comments and conversations that improved the study and the manuscript. C. Schwarz provided helpful statistical advice. S. Chen, C. Gerak, and E. Wu assisted with spider rearing and maintenance.

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Table 3.1 Summary statistics describing the number of *Latrodectus hesperus* males captured on adhesive strips around wire mesh cages during the 24-h field trapping experiment 1 commencing at 2100 h on 21 August 2013. The four cages within each of the 20 experimental replicates (N) were randomly assigned one of the four treatments listed in the table

Treatments	N	First 6 h (2100 - 0300 h)				First 12 h	24 h
		Mean	Median	Range	Total	Total	Total
Intact web	20	3.80	3.5	0 - 13	76	103	105
Male-reduced web	20	1.05	0	0 - 5	21	34	36
Mechanically reduced web	20	2.65	2	0 - 8	53	82	83
No web	20	0.25	0	0 - 2	5	5	6
Total	80	1.94	1	0 - 13	155	224	230

Table 3.2 Summary statistics describing the number of *Latrodectus hesperus* males captured on adhesive strips around cages during the 24-h field trapping experiment 2 commencing at 2030 h on 9 September 2013. The two cages within each of the 20 experimental replicates (N) were randomly assigned one of the two treatments listed in the table

Treatments	N	First 6 h (2030 - 0230 h)				First 12 h	24 h
		Mean	Median	Range	Total	Total	Total
Intact web	20	7.05	7.5	0 - 21	141	192	242
Intact web plus male silk ^a	20	6.35	5.5	0 - 21	127	189	219
Total	40	6.70	6.5	0 - 21	268	381	461

^aEntire female's web plus an unknown quantity of a second female's web bundled and wrapped with male silk

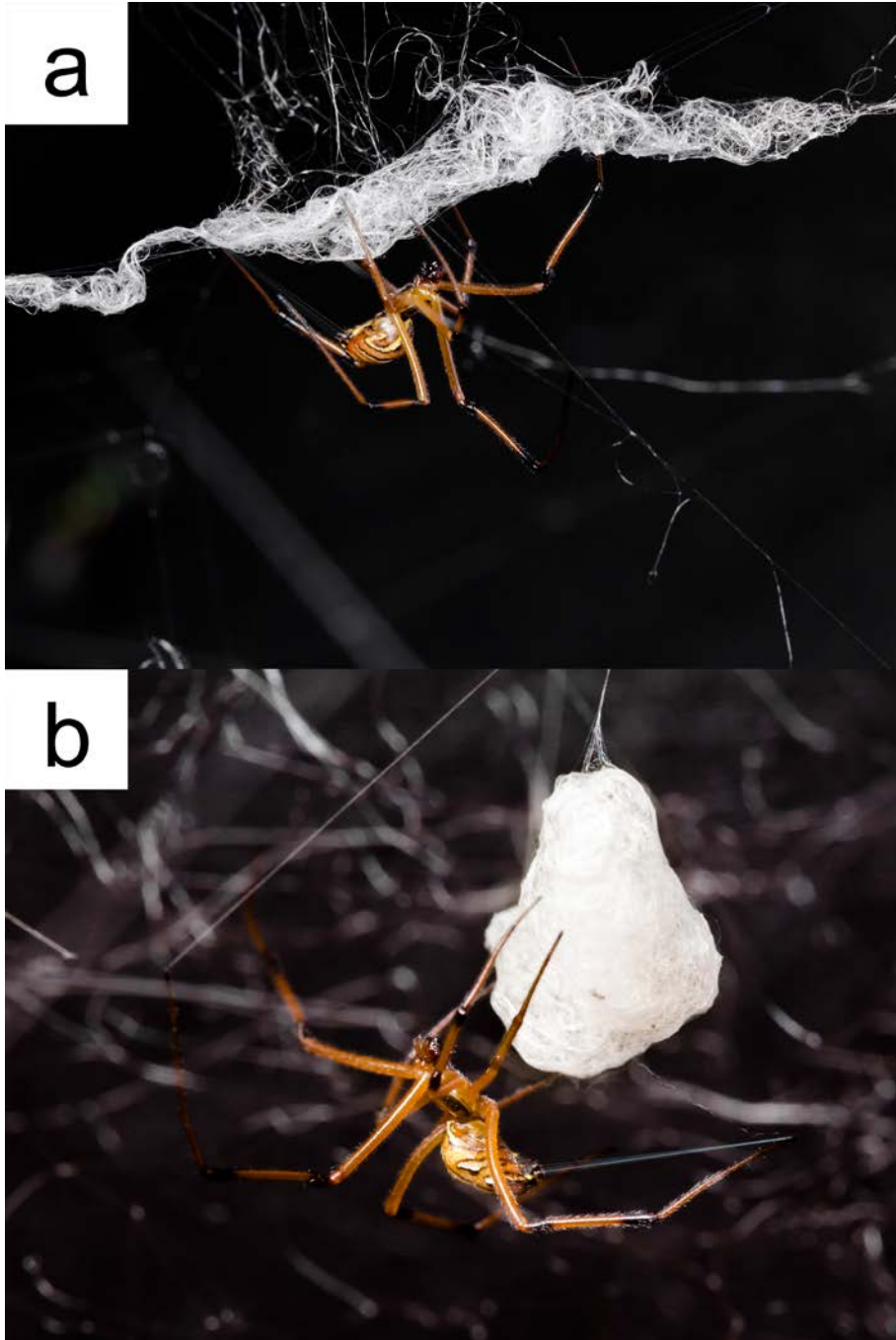


Figure 3.1 Photographs of male *Latrodectus hesperus* during web reduction. During pre-copulatory courtship on the female's web the male excises sections of silk, bundles them up, and wraps them with his own silk, resulting in a rope (a) or ball (b) of silk. Note that in each image the male is pulling wrapping silk from his spinnerets with the last pair of legs.

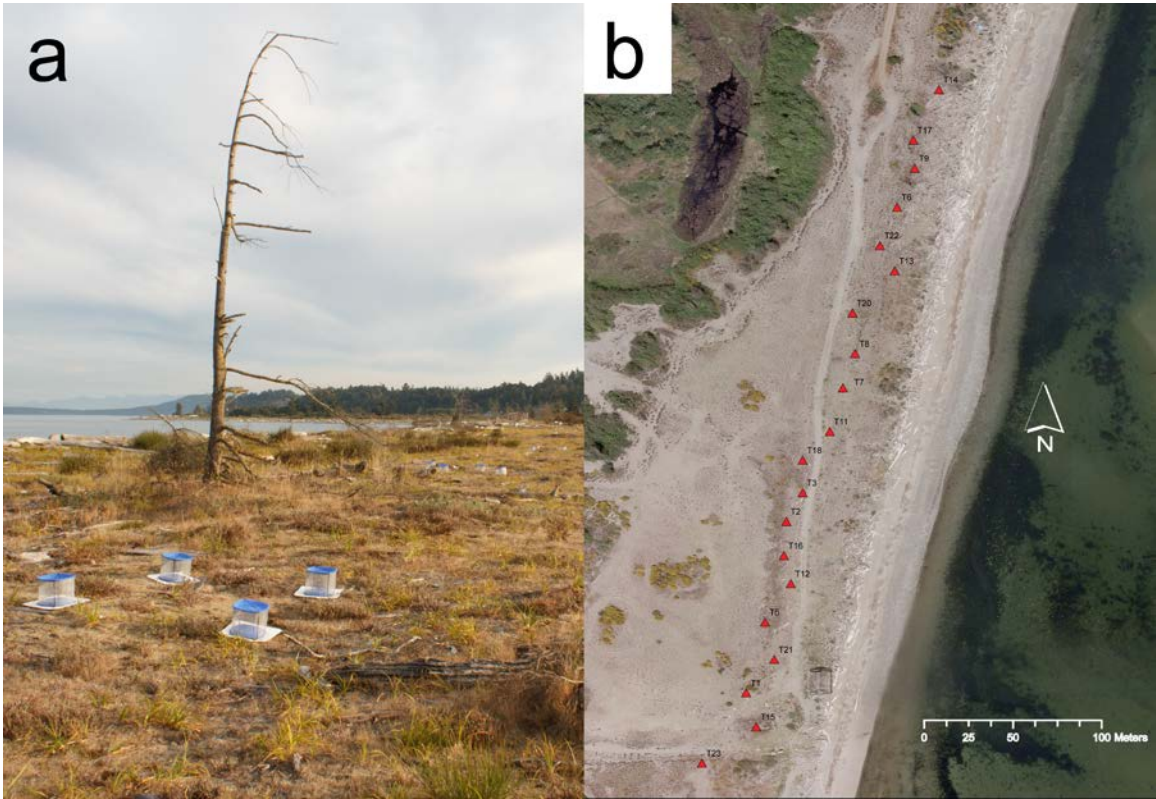


Figure 3.2 (a) Photograph of one of 20 blocks tested in experiment 1, illustrating four wire mesh cages surrounded by adhesive strips and spaced 1 m apart (see methods for details of experimental design). (b) Aerial view of the Island View Beach field site on the Saanich Peninsula of Vancouver Island, BC, Canada (image courtesy of the Capital Regional District). Red triangles indicate the location of experimental replicates placed approximately 20 m apart in experiments 1 and 2.

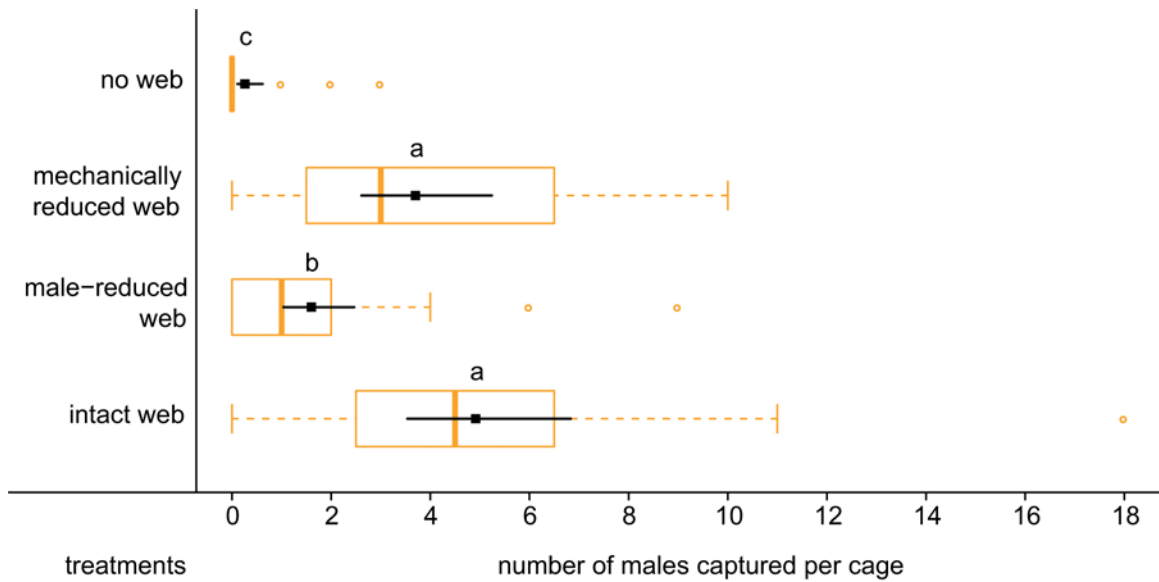


Figure 3.3 The number of *Latrodectus hesperus* males attracted over 24 h to wire mesh cages containing (1) intact webs of virgin females, (2) experimentally reduced webs (with 50% of the silk removed by us), (3) male-reduced webs (with on average 50% of the silk bundled up and wrapped with silk by a courting male), and (4) empty controls in a randomized complete block field trapping experiment with 20 replicate blocks. Orange box plots display the raw data (box represents the interquartile range (IQR), whiskers extend to 1.5 times the IQR, horizontal lines show the median, and open circles are outliers). Back-transformed marginal means and 95% confidence intervals from a negative binomial GLM including the effects of treatments and blocks are shown in black. Different letters indicate means that are significantly different from one another ($P < 0.05$ after adjusting for multiple comparisons using the Tukey method).

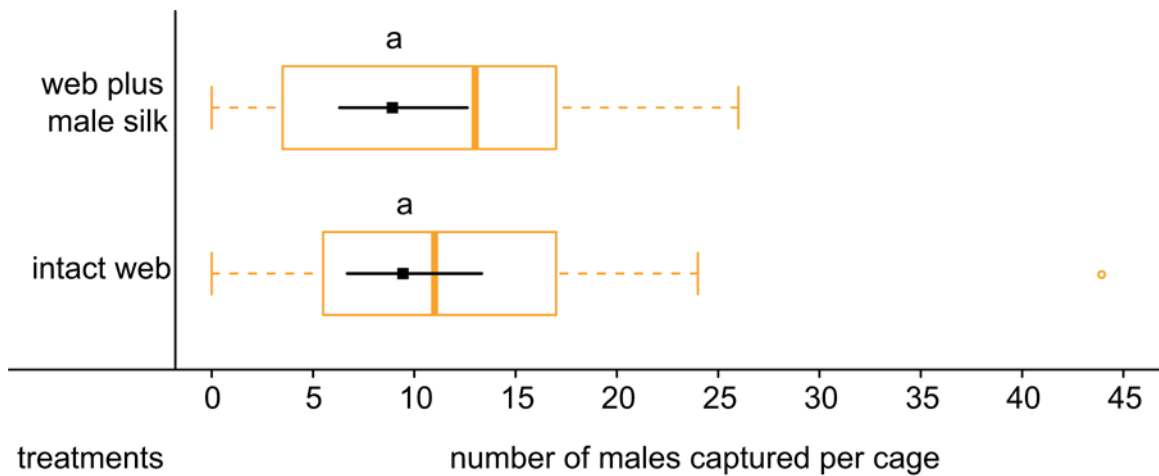


Figure 3.4 The number of *Latrodectus hesperus* males attracted over 24 h to wire cages containing (1) intact webs of virgin females and (2) intact webs of virgin females with a male silk-wrapped bundle or ball from a separate reduced web in a randomized complete block field trapping experiment with 20 replicate blocks. Orange box plots display the raw data (box represents the interquartile range (IQR), whiskers extend to 1.5 times the IQR, horizontal line shows the median, and open circles are outliers). Back-transformed marginal means and 95% confidence intervals from a negative binomial GLM including the effects of treatments and blocks are shown in black. The same letters indicate that the means are not significantly different from one another ($P = 0.74$).

Appendix A Supplementary Material for Chapter 1

Video A1

Creator:

Samantha Vibert

Description:

A male black widow (*Latrodectus hesperus*) wraps a female with a silk 'bridal veil' during pre-copulatory courtship. He uses the last two pairs of legs to pull silk from his spinnerets and deposits it on the female. Note the silk threads of the veil already visible across the female's abdomen.

Filename:

Video_A1.mp4

Appendix B Supplementary Material for Chapter 2

Chemical Analyses

General Methods and Instrumentation for Syntheses

We hot assembled oven-dried glassware under Ar flow, and maintained it under Ar. We transferred liquids by cannula under Ar pressure and obtained NMR spectra of synthetic compounds on a Bruker BioSpin-400 spectrometer (Bruker, Rheinstetten, Germany) (at 400 MHz for ^1H , 100 MHz for ^{13}C) with CDCl_3 as a solvent; we report chemical shifts in ppm relative to TMS (^1H , δ 0.00) and CDCl_3 (^{13}C , δ 77.00).

Representative Synthesis of Ester-Amide Mixtures

We mixed anhydrous K_2CO_3 (20 mg; 1.5 mmol) with *L*-serine methyl ester hydrochloride (100 mg; 0.65 mmol) (Sigma, St. Louis, MO 63103, USA) in dichloromethane (3 ml) and then added 2-methylbutyric acid (60 ml; 0.65 mmol) (Aldrich, Milwaukee, WI, 53201, USA) and isobutyric acid (50 ml; 0.65 mmol) (Aldrich), followed by addition of *N,N'*-dicyclohexylcarbodiimide (0.54 g; 4 equivalents (Aldrich) and DMAP (2 mg). After stirring the mixture for 6 h, we separated products from urea and unreacted materials by flash chromatography using ethyl acetate as eluent. We concentrated the ethyl acetate solution *in vacuo*, and added ether. We removed insoluble precipitate by filtration and concentrated the filtrate, yielding 90 mg of a mixture containing di-acylated compounds (70%, GC).

Synthesis of N-3-methylbutyryl-O-methylpropanoyl-L-serine methyl ester (Fig. 1)

We added isobutyric anhydride (9.12 mmol; 1.51 ml, Aldrich) to *N*-(*tert*-butoxycarbonyl)-*L*-serine methyl ester (0.5 g; 2.28 mmol) (**1**) (Sigma). While stirring the reaction mixture, we added anhydrous potassium carbonate (1.26 g, 9.12 mmol) and DMAP (2 mg), and then continued to stir for 48 h at ambient temperatures. We extracted

products with ether (2 × 50 ml), and washed extracts with saturated aqueous sodium bicarbonate solution, water, and brine. After drying (MgSO₄, anh.), we concentrated extracts *in vacuo*. We purified *N*-(Boc)-*O*-isobutyryl-*L*-serine methyl ester (**2**) by flash chromatography with 25% ether in hexane as eluent. Quantitative yield (99% pure, GC). ¹H NMR (400 MHz, CDCl₃): δ 5.27 (d, *J* = 7.8 Hz, 1H), 4.58 (m, 1H), 4.45 (dd, *J* = 11.2, 4.0 Hz, 1H), 4.29 (dd, *J* = 11.3, 3.6 Hz, 1H), 3.75 (s, 3H), 2.54 (hept, *J* = 7.0 Hz, 1H), 1.45 (s, 9H), 1.14 (dd, *J* = 7.0, 3.1 Hz, 6H). ¹³C NMR (CDCl₃, 100 MHz): 176.5, 170.3, 155.1, 80.3, 64.0, 53.02, 52.6, 33.8, 28.3, 18.9, 18.8. We removed the protective carbamate group from **2** by treating it with excess of trifluoric acid (5 ml) in dichloromethane (25 ml) at room temperature for 1 h. We removed solvents *in vacuo*, and used the crude mixture without purification for *N*-acylation. To this effect, we proceeded at 0 °C with dropwise addition of 0.95 ml (7.80 mmol) of isovaleryl chloride (Aldrich) to the stirred solution of *O*-isobutyryl-*L*-serine methyl ester and triethylamine (2.0 ml, 14.3 mmol) in dichloromethane (10 ml). We warmed the reaction mixture to room temperature and extracted it with ether (3 × 25 ml). We then washed the extract with 2N aqueous HCl, saturated sodium bicarbonate solution, and brine. After drying the extract with anh. MgSO₄, we filtered and concentrated it, and purified the final product by column chromatography with hexane, and hexane/ether (5:95 and 50:50%) as consecutive eluents. Yield of the pure di-acylated serine ester **3** was 0.55 g (2.01 mmol, 88% yield). ¹H NMR (400 MHz, CDCl₃): δ 6.22 (d, *J* = 7.2 Hz, 1H), 4.86 (dt, *J* = 7.6, 3.7 Hz, 1H), 4.47 (dd, *J* = 11.4, 4.0 Hz, 1H), 4.34 (dd, *J* = 11.4, 3.4 Hz, 1H), 3.76 (s, 3H), 2.53 (hept, *J* = 7.0 Hz, 1H), 2.09-2.12 (m, 3H), 1.13 (dd, *J* = 7.0, 2.4 Hz, 6H), 0.93-0.98 (m, 6H). ¹³C NMR (CDCl₃, 100 MHz): 176.6, 172.2, 170.1, 63.7, 52.7, 51.8, 45.7, 33.8, 26.1, 22.4, 22.3, 18.9, 18.8. HREIMS: *m/z* calcd. for C₁₃H₂₄NO₅ [M+H]⁺ 274.1649,

found 274.1641; calcd. for $C_{13}H_{23}NNaO_5$ $[M+Na]^+$ 296.1468, found 296.1460; calcd. for $C_{13}H_{23}KNO_5$ $[M+K]^+$ 312.1208, found 312.1203.

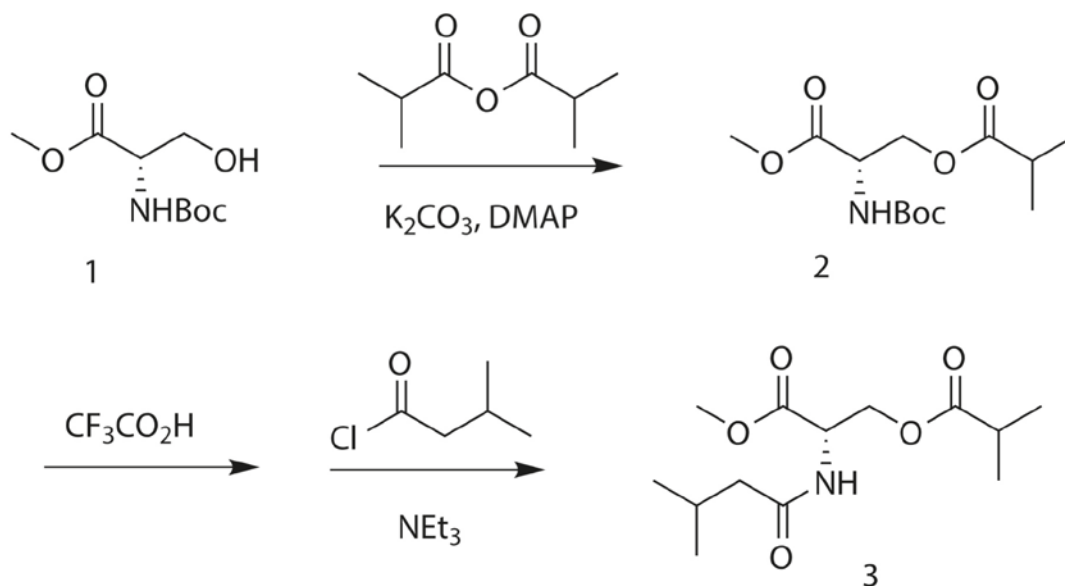


Figure A1 Synthesis of *N*-3-methylbutyryl-*O*-methylpropanoyl-*L*-serine methyl ester (3), candidate pheromone component of female *Latrodectus hesperus*; DMAP = 4-dimethylaminopyridine

Video B1

Creator:

Sean McCann

Description:

A Latrodectus hesperus male silk-wrapping a filter paper treated with methanol extract of a virgin female's web, first at full speed, then slowed to half-speed.

Filename:

Video_B1.mp4