Function and diversity of sex pheromones in representative species of the black widow spiders (genus Latrodectus, Araneae: Theridiidae)

by

Luciana Baruffaldi

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Ecology and Evolutionary Biology
University of Toronto

© Copyright by Luciana Baruffaldi 2016
Function and diversity of sex pheromones in representative species of the black widow spiders (genus Latrodectus, Araneae: Theridiidae)

Luciana Baruffaldi

Doctor of Philosophy

Ecology and Evolutionary Biology

University of Toronto

2016

Abstract

Understanding causes and consequences of signal diversity can give insight into how individual fitness is affected by communication, and how this relates to processes from species diversification to mate preferences. During my PhD, I first examined functional divergence in sex pheromones in widow spiders (Theridiidae: Latrodectus), focusing on six species representing different clades and biogeographic regions. I tested the hypothesis that divergence in sex pheromones was predicted by phylogenetic divergence (measured as CO1 sequence dissimilarity). I showed that the most distantly related of my focal species responded only to pheromone extracts from the silk of conspecifics; whereas males from the other species also responded to extracts from more closely related heterospecifics, and, as predicted, male response and genetic divergence were negatively correlated. Next, focusing on two focal Latrodectus species with different mating systems, I showed that males can use sex pheromones alone to discriminate among females of different feeding conditions. Inter-
specific differences in male discrimination suggested that preferences may arise from benefits of avoiding risky matings with potential cannibals, rather than from seeking fecund, well-fed females. Finally, I examined one *Latrodectus* species with a recently-demonstrated, novel male mating tactic (mating with immature females), asked whether the tactic is likely to be costly for females, and whether it affects female mate-attraction tactics (pheromone production). I found no cost of immature-mating for females in terms of fertility, fecundity or longevity. Moreover, I showed that immature-mated females do not produce sex pheromones after moulting and so would not attract additional mates after moulting, unlike recently-moulted females that are unmated. My research shows how sex pheromone function varies among species and individuals with different ecologies and mating behaviours, and how this may affect individual fitness and mating decisions, yielding intriguing new insights into how sexual signals are affected by speciation and sexual selection.
Acknowledgments

A doctoral thesis requires enormous discipline and hard work. The fact that I completed my thesis not only in another language, but also in a country with a culture and climate so different from my own, was a huge personal challenge, and my success was made possible by the unconditional support of several people to whom I am deeply grateful.

First of all I would like to thank my amazing supervisor, Maydianne Andrade, for everything that I have learned as her student - about science, mentorship, and most importantly, about growing as a more compassionate human being. Since we met and I joined her laboratory her support has been unconditional at all levels. She is one of the smartest people I have ever met, and is also one with the most humble character demonstrated in the way that she interacts with students and peers. Her level of support surpassed my expectations as she taught me new things and helped me with the problems that I faced during the course of my PhD. Maydianne gave me the space and independence to develop my own ideas at my own pace. Without her constant support, it would not have been possible for me to include as many species in my research, have the opportunity to travel to different regions to collect them, or present my results at numerous conferences during the course of my PhD tenure. She has not only been my mentor, but someone with whom I could share other personal aspects of my life and I hope that our friendship will continue into the future.

I would also like to thank my lab partners Sheena Fry, Charmaine Condy, Emily MacLeod, Maria Modanu and Monica Mowery for always being at my side helping me with my papers, and presentations and contributing to rich discussions about the direction and impact of my projects. Their friendships and support through the years have been invaluable. I want to especially thank Charmaine Condy for her unconditional support and collaboration with the genetic analysis in Chapter 2 of the thesis and for hugging me every day; and Maria Modanu for her friendship and for making me understand that good friends can be found in any place and time in life.

I would also like to thank the members of my PhD Committee, Darryl Gwynne and Jason Weir, for their support, availability, comments and suggestions during the course of my tenure which had a great impact in defining my projects.

I am grateful to Emily MacLeod, Sheena Fry, and Michael Kasumovic for collecting and providing spiders from California and Australia. To all of my laboratory and field assistants over the years, without your collaboration it would have been very difficult to take care of all the spider populations. I want to especially thank Humera Siddiqui and Athithya Thambiappah for their help with the volatile pheromone experiments. Also to all the volunteers and laboratory managers, thank you for your very important role in growing and maintaining our spider populations.

I would like to especially thank all the members of the Laboratory of Ethology, Ecology and Evolution (IIBCE-Uruguay) for all their unconditional support over the years, especially to Laura Montes de Oca for her help with L. mirabilis in the field and in the laboratory, and Anita Aisenberg and Fernando Costa for their affection and always allowing me to develop experiments in the laboratory. I am grateful to the “Direccion General de Recursos Naturals Renovables-Division de Areas Protegidas y Fauna- Departamento de Fauna (Montevideo-Uruguay)” for allowing me to collect and export L. mirabilis from Uruguay.
I am grateful to the financial support from the Animal Behaviour Society, The Society of Integrative and Comparative Biology, and the “Comision Sectorial de Investigacion Cientifica (Uruguay)”. 

To my fellow Latinos at UofT!! Paola, Santiago, Maya, Alfredo, Eli, Santiago, Sol, thanks for the moments we shared together including parties, meals, and nights out that made me feel at home during all of the times that we spent together. I want to also thank Mauricio Terebiznik and his family for opening their home to us all.

To my friends back home, they made me feel closer than ever and were always by my side, just only a call away listening and encouraging me at every step, Sol, Naty, Lu, Carla, Magy, Lali, Lili and Lau.

To my family, especially my parents for whom it was not easy to have me so far from home, but always unconditionally supporting my studies. Since a very young age, they taught me the importance of having the best education possible in order to have more opportunities in life and be able to choose what makes you happy, even if it meant to travel so far and work with poisonous spiders and not cuter animals.

To Mar, for being always by my side, in the lowest and highest moments of our journey together, for caring so much when I needed you the most, and for always looking on the bright side of things. Without your support, friendship, understanding and love nothing would have been easier!

To Bicha….

Thanks,

Lu

Agradecimientos

Una tesis de doctorado requiere de mucha disciplina, esfuerzo y trabajo. Especialmente para mí, hacerla no solamente en otro idioma sino también en un país con una cultura y clima tan diferente, fue un desafío personal muy grande y fue posible por el apoyo incondicional de varias personas a las cuales les estoy profundamente agradecida.

Primero que nada me gustaría agradecerle a mi maravillosa supervisora, Maydianne Andrade, por todo lo que he aprendido siendo su estudiante sobre ciencia, tutelaje y también sobre crecer como un ser humano mas compasivo. Desde que nos conocimos y me uní a su laboratorio, su apoyo ha sido incondicional a todos los niveles. Ella es una de las personas más inteligentes que he conocido, y también es una de las más humildes en su manera de interactuar con sus estudiantes y pares. Su nivel de apoyo fue más allá de mis expectativas a la hora de enseñarme y ayudarme con los problemas a los que fui enfrentándome durante el transcurso de mi doctorado. Maydianne me dio la independencia para poder desarrollar mis propias ideas y a mi propio ritmo. Sin su constante apoyo no hubiese sido posible para mí incluir numerosas especies en mi tesis, ni poder viajar a colectarlas a diferentes regiones, como tampoco poder presentar mis resultados en numerosos congresos durante el transcurso de mi doctorado. No solo ha sido mi mentora, sino alguien con quien pude compartir otros aspectos de mi vida y espero que nuestra amistad continúe por muchísimo tiempo.

También me gustaría agradecerles a mis compañeras de laboratorio Sheena Fry, Charmaine Condy, Emily MacLeod, Maria Modanu and Monica Mowery por estar siempre a mi lado
ayudándome con mis presentaciones, papers y en numerosas charlas acerca de la dirección e impacto de mis proyectos. Su amistad y apoyo en todo este tiempo ha sido invaluable. Quiero especialmente agradecerle a Charmaine Condy por su ayuda incondicional y colaboración en el capítulo 2 de la tesis y por abrazarme todos los días; y a María Modanu por su amistad y por hacerme entender que muy buenos amigos se pueden encontrar en cualquier lugar y momento de la vida.

A mi comité de seguimiento Darryl Gwynne y Jason Weir, por su apoyo, disposición, comentarios y sugerencias a lo largo del transcurso de mi doctorado los cuales tuvieron un gran impacto a la hora de definir mis proyectos.

Le estoy agradecida a Emily MacLeod, Sheena Fry and Michael Kasumovic por colectar y procurar species desde California y Australia. A todos mis asistentes de laboratorio y de campo durante estos años, sin su colaboración hubiese sido muy difícil el cuidado de todas las poblaciones. Quiero agradecerle especialmente a Humera Siddiqui y Athithya Thambiappah por su ayuda en los experimentos de feromonas volátiles. También a todos los voluntarios y managers por su invaluable ayuda en el criado de las poblaciones.

Me gustaría especialmente agradecerles a tod@s l@s integrantes del Laboratorio de Etología, Ecología y Evolución del IIBCE-Uruguay por todo su apoyo incondicional durante estos años, especialmente a Laura Montes de Oca por su ayuda con L. mirabilis, Anita Aisenberg y Fernando Costa por su cariño y por siempre permitirme desarrollar experimentos en el laboratorio. A la Dirección General de Recursos Naturales Renovables-División de Áreas Protegidas y Fauna- Departamento de Fauna (Montevideo-Uruguay), por permitirme colectar y exportar L. mirabilis desde Uruguay.

También me gustaría agradecerle a la “Animal Behaviour Society”, “The Society of Integrative and Comparative Biology”, y a la Comisión Sectorial de Investigación Científica (Uruguay) por su apoyo financiero.

A mis compañero@s latin@s en UofT!! Paola, Santiago, Maya, Alfredo, Eli, Santiago, Sol, gracias a su cariño y los momentos que compartimos juntos, fiestas, salidas, comidas, hicieron que tuviera un poquito de mi cultura aun estando lejos, y que me sintiera como en casa en cada momento que pasamos juntos. Quiero aprovechar e incluir a Mauricio Terebiznik y su familia por haber abierto las puertas de su hogar a tod@s nostvr@s.

A mis amigas del alma, las cuales me hicieron sentir más cerca que nunca y siempre estuvieron a mi lado, a una llamada a la distancia escuchándome y alentándome a cada paso, Sol, Naty, Lu, Carla, Magy, Lali, Lili, Lau.

A mi familia, principalmente a mis padres para los cuales no fue fácil tenerme tan lejos de casa, pero siempre me apoyaron incondicionalmente con mis estudios. Desde muy chica me hablaron de la importancia de poder tener la mejor educación posible para poder tener más oportunidades y poder elegir lo que te hiciera feliz en la vida, aunque eso significara irme tan lejos y trabajar con arañas venenosas en vez de animalitos más lindos.

A Marcela, por estar siempre a mi lado, en las buenas y en las malas, por cuidarme tanto cuando más te necesite y estar mirando siempre el lado positivo de las cosas. Sin tu apoyo, compañerismo, entendimiento y amor nada hubiese sido tan fácil!

A Bicha……

Muchas gracias,

Lu
# Table of Contents

Abstract ........................................................................................................................................... ii
Acknowledgments ............................................................................................................................... iv
Table of Contents .............................................................................................................................. vii
List of Tables ....................................................................................................................................... xi
List of Figures ...................................................................................................................................... xii
Chapter 1 ........................................................................................................................................... 1
  General Introduction ........................................................................................................................ 1
    Signal diversity and species diversification ............................................................................... 2
    Signal diversity and individual variation ............................................................................... 5
    Chemical signals ....................................................................................................................... 7
    Sex pheromones: mate attraction, localization, and courtship ........................................... 10
    Sex pheromones & mate choice ............................................................................................. 11
    Chemical signals in spiders .................................................................................................. 13
    Inter-specific patterns ............................................................................................................ 14
    Intra-specific patterns ............................................................................................................. 16
    Potential impact of comparative study of spider pheromone function .................................. 20
    Thesis goals ............................................................................................................................ 21
Latrodectus Natural History ........................................................................................................... 22
Ecology and Behaviour .................................................................................................................. 22
Chemical communication in Latrodectus .................................................................................... 25
Intra-specific variation ................................................................................................................... 26
Inter-specific variation ................................................................................................................... 27
Focal species ................................................................................................................................. 28
Overview of Chapters .......................................................................................................................... 33
References ......................................................................................................................................... 37

Chapter 2 ........................................................................................................................................... 52
Phylogenetic pattern of sex pheromone discrimination in spiders: evidence from widow spiders ................................................................. 52
Abstract .............................................................................................................................................. 52
Introduction ....................................................................................................................................... 54
Methods ............................................................................................................................................. 60
Latrodectus biology & natural history ............................................................................................... 60
Experimental Spiders ....................................................................................................................... 61
Part 1. Contact pheromone bioassay ............................................................................................... 62
Part 2. Airborne pheromones .......................................................................................................... 64
Part 3. Laboratory matings .............................................................................................................. 65
Part 4. Genetic Distance and behaviour .......................................................................................... 65
Statistical analysis ............................................................................................................................. 66
Results ............................................................................................................................................... 66
Part 1. Contact pheromones ............................................................................................................ 66
Part 2. Airborne pheromones .......................................................................................................... 67
Part 3. Matings with conspecific and heterospecific females ........................................................... 68
Part 4. Genetic Distance and behavior ............................................................................................ 68
Discussion .......................................................................................................................................... 69
References .......................................................................................................................................... 75

Chapter 3 ........................................................................................................................................... 89
Contact pheromones mediate male preference in black widow spiders: avoidance of hungry sexual cannibals? ................................................................. 89
Experimental females ................................................................. 130
Mating trials ................................................................................. 130
Direct fitness effects of mating ....................................................... 131
Silk collection and pheromone extraction ....................................... 131
Sex pheromone bioassay ................................................................. 132
Statistical analysis ........................................................................ 133
Pre-copulatory interactions and Mating outcomes .......................... 133
Direct fitness effects of immature mating ...................................... 134
Sex pheromone bioassay ................................................................. 134
Results ......................................................................................... 134
Pre-copulatory interactions ............................................................ 134
Mating outcomes .......................................................................... 136
Direct fitness effects of immature mating for females .................. 137
Sex pheromone bioassay ................................................................. 137
Discussion .................................................................................... 138
References ................................................................................... 148
General Discussion ....................................................................... 161
Future Directions .......................................................................... 167
Conclusion .................................................................................... 170
References ................................................................................... 172
Appendix 1 ..................................................................................... 177
Appendix 2 ..................................................................................... 181
Appendix 3 ..................................................................................... 185
List of Tables

Table 2-1 .......................................................................................................................... 87
Table 2-2 .......................................................................................................................... 88
Table 3-1 .......................................................................................................................... 114
Table 4-1 .......................................................................................................................... 154
Table 4-2 .......................................................................................................................... 155
Table A-3-1 ....................................................................................................................... 185
List of Figures

Figure 1-1 .......................................................................................................................... 51
Figure 2-1 .......................................................................................................................... 82
Figure 2-2 .......................................................................................................................... 83
Figure 2-3 .......................................................................................................................... 84
Figure 2-4 .......................................................................................................................... 85
Figure 2-5 .......................................................................................................................... 86
Figure 3-1 .......................................................................................................................... 116
Figure 3-2 .......................................................................................................................... 117
Figure 3-3 .......................................................................................................................... 118
Figure 4-1 .......................................................................................................................... 157
Figure 4-2 .......................................................................................................................... 158
Figure 4-3 .......................................................................................................................... 159
Figure 4-4 .......................................................................................................................... 160
Figure A-1-1 ...................................................................................................................... 179
Figure A-1-2 ...................................................................................................................... 180
Figure A-2-1 ...................................................................................................................... 183
Figure A-2-2 ...................................................................................................................... 184
Chapter 1

General Introduction

Communication, the exchange of information between individuals, is a critical part of a wide range of behavioural interactions such as competition, mating, aggregation, and cooperation (Bradbury & Vehrencamp, 2011). Signals are cues that evolve to efficiently transmit information to receivers if that exchange is beneficial to the sender (Bradbury & Vehrencamp, 2011; Wilson, 1975). There are many levels of signal diversity, as signals may vary within and among individuals within a species (e.g., state or context-dependent signal production), and also among species (e.g., variation in signal modality or components). This diversity can have important consequences for patterns of behavioural interactions (Edward & Chapman, 2011; Symonds & Elgar, 2008; Thomas, 2011) and understanding sources and consequences of signal diversity can give insight into how individual fitness is affected by communication (Thomas, 2011), but also how this relates to processes initiating, mediating, or following from species diversification (Symonds & Elgar, 2008; Wilkings et al., 2013).

My PhD focuses on understanding factors affecting the diversity and function of signals at two levels (among species and within individuals) in a relatively poorly studied signal modality (chemical signals), in a sparsely studied taxon (spiders). My work is aimed at testing general hypotheses about signal diversity in ways that capitalize on unique aspects of this modality and taxon.
**Signal diversity and species diversification**

The biological species concept defines species as groups of natural populations that are capable of interbreeding and that are reproductively isolated from other groups (Mayr, 1942). New species arise when populations that once interbred diverge and become unlikely to exchange genes successfully with each other (Patterson, 1985; Venditti & Pagel, 2009). Divergence may occur via selection or drift in populations that are geographically isolated (e.g. events of allopatry, Cook, 1906). For populations in sympatry, divergence can arise via adaptation to different environments or niches (e.g., for example ecological character displacement, Gourbiere, 2004), direct selection on mate preferences (e.g., if individuals mate assortatively, Higashi et al., 1999; Nosil et al., 2006), or by reinforcement if hybrids are less fit than parental types (Albert et al., 2004; Dobzhansky, 1940). Sexual signals have been the focus of many classic studies of species divergence (Andersson, 1994; Coyne & Orr, 2004). Animals are surrounded by signals emitted from conspecifics, heterospecific congeners, and more distantly related species. Since sexual signals are important mediators of mate attraction and mating decisions in many taxa, selection driven by mate preference or reproductive isolation may lead to significant differences in the signals of closely related species (Anderson, 1994; Arnqvist & Rowe, 2005). In addition, overlap in signaling with other species could reduce the effectiveness of a signal, impairing communication between the sender and receiver, hence decreasing their fitness (Anderson, 1994; Arnqvist & Rowe, 2005; Higgie & Blows, 2007; Wilkings et al., 2013). Therefore, it is predicted that mechanisms will evolve that allow receivers to distinguish signals or cues that are relevant, and/or senders will evolve to produce signals that avoid overlap with heterospecific signals.
(Hebets & Papaj, 2005; Higgle & Blows, 2007). This will be particularly acute in zones of sympatry when there is a cost to making a mating mistake. Thus for related species found in sympatry, differences in the signals involved in pre-mating interactions are expected to accumulate (Brown & Wilson, 1956; Dobzhansky, 1937). In sympatric areas, both sexual selection through mate preference (intraspecific), and species recognition, the ability to choose a mate of your own species (interspecific), will act together (Dobzhansky, 1951; Howard, 1993). This effect has been found across signal modalities. For example the three sympatric species of European ermine moths (*Yponomeuta*) that share the same host-plant showed distinct species-specific ratios of components of their sex pheromones, which minimizes the risk of hybridization (Lofstedt et al., 1991). This differentiation could be due to reproductive character displacement upon secondary contact between species that have been already diverged genetically in allopatry. Moreover, in Caribbean *Anolis* lizards, closely related parapatric species differed more in their dewlap phenotypes (colouration) than those that were allopatric and more distantly related, and this facilitates species recognition in sympatry (Ng et al., 2013). Traits of the receiver may also shift in response to the risk of hybridization. For example in two species of the genus *Alytes* (midwife toads) females prefer long-duration calls when in allopatry. However there is considerable overlap in the call durations of *Alytes obstetricans* and *A. cisternasii* males, with *A. obstetricans* males making shorter calls on average. When these species are found in sympatry, *A. obstetricans* females no longer show a preference for long calls, demonstrating the reproductive character displacement in their preference (Marchez & Bosch, 1997).

In contrast to these examples, when there is low risk of interbreeding between related species, or species rely on other communicatory channels for species identification, we
expect to observe less differentiation between closely related species. In these cases, there may be a strong positive relationship between phylogenetic distance and signal diversity (Bradbury & Vehrencamp, 2011; Butlin, et al., 2012; Kirkpatrick & Ryan, 1991; Otte & Endler, 1989; Philips & Johnston, 2009; Wilson, 1975). For example, in the shiner fish *Cyprinella*, sister species *C. gibbsi* and *C. trichroistia* exhibit more similar components in their courtship call repertoire than the more distantly related *C. galactura* and *C. callisema* (Phillips & Johnston, 2009). In this example, sister species are currently allopatric. If this were also true historically, then there would have been no selection for reproductive character displacement. This pattern may also arise if any historical effects of sympatry are lost in currently allopatric populations (McLennan & Ryan, 1997).

For many taxa, it is well-established that divergence occurs in allopatry, when gene flow is severed by some physical barrier or dispersal event, and divergence of populations is mainly driven by genetic drift with geographic isolation (Butlin et al., 2008; Cracraft, 1989; Coyne & Orr, 2004). Reinforcement of pre-existing differences (i.e., reproductive character displacement) may also occur after secondary contact if approaching heterospecifics is sufficiently costly, and historically, it was thought that selection on females would primarily drive these effects (Parker & Patridge, 1998). Parker and Partridge (1998) predicted that in the event of secondary contact, females would typically exhibit stronger discrimination between different population or species, because their higher parental investment would make hybridization more costly for females than males (Arnvist & Rowe, 1995; Chapman et al., 1995; Fowler & Patridge, 1998). In comparison, for males, the risk of making a mistake and forgoing a viable mate may make strict discrimination thresholds less likely (e.g. Noor, 1996; Parker & Partridge, 1998). However, more recently, it has been recognized that
males that invest time and energy searching for and courting heterospecific females could be constraining their future mating opportunities (Edward & Chapman, 2011), and thus selection for male discrimination may be significant (Andersson, 1994; Edward & Chapman, 2011). Consistent with this, evidence for male mate choice in general and for male discrimination between conspecific and heterospecific females has been accumulating (Barbosa et al., 2006; Ciccotto et al., 2013; Edward & Chapman 2011; Espinedo et al., 2010; Wong et al., 2005).

**Signal diversity and individual variation**

The problem of choosing an appropriate mate includes not only whether a signaler is a conspecific, but also whether a particular conspecific is suitable or receptive. Such information may be present in concert with information about species identity. Signals may then include features that vary across species but are static within species, in combination with dynamic features that are context- or condition-dependent and vary within species (Andersson, 1994; Bradbury & Vehrencamp, 2011). Static signals (or signal components) are expected to be highly stereotyped in structure within individuals from the same species, such that static features determine the quality of the signal (e.g., evolved variation in signal components or sequence). Static signals would thus be under stabilizing selection via species recognition. Co-evolution of signal structure (in senders) and the sensory systems specialized to detect that structure (in receivers) is expected, with individuals that deviate from the population-mean range paying a fitness cost (Gerhardt 1988, 1991; Charalambous et al., 1994; Ritchie & Kyriacou, 1994; Ryan & Rand, 1993). In contrast, dynamic signals (or signal components) are predicted to be highly variable within species, and traits of
individuals are expected to determine the quantity of dynamic signaling (e.g., in treefrogs females prefer conspecific males producing long calls with high rates rather than short calls with low rates Gerhardt, 1991). Dynamic signal components are often correlated with fitness-related traits (e.g. male body size or condition; female fecundity, reproductive status or condition), and are expected to be good indicators of the quality or receptivity of a potential partner (Kotiaho et al., 1996; Zahavi, 1975). Thus dynamic signals may frequently be under directional selection, with more extreme signals preferred by potential mates (Aspi & Hoikkala, 1995; Bailey et al., 1990; Hedrick, 1986; Jang & Greenfield, 1996; Simmons, 1988; Tuckerman et al., 1993). For example, studies in junglefowl (Gallus gallus) showed that females preferred males with longer, more brilliantly colored combs and redder feathers, which are indicators of male health (Zuk et al., 1990). In the wolf spider H. rubrofasciata females prefer males with the highest drumming rate (Kotiaho et al., 1996; Parri et al., 1997), which is highly variable among males, is costly energetically, and affects male survival (Kotiaho et al., 1998 a,b; Mappes et al., 1996).

Males’ calling songs and visual displays have played a key role in understanding effects of static and dynamic signals on mate attraction and receptivity. This is likely because these are generally sexually dimorphic, easily detectable (for females and for researchers), and are highly variable and energetically expensive to produce (Andresson, 1994; Basolo & Trainor, 2002; Brown et al., 1996; Searcy & Andersson, 1986; Zuk et al., 1990). However, it is clear that both females and males use information encoded in dynamic signals to discriminate and chose among potential conspecific mates (Andersson, 1994; Edward & Chapman, 2011; Trivers, 1972). Thus, coincident with the increased appreciation for the importance of male mate choice in general (Bonduriansky, 2001), more studies have focused
on the role of female-produced signals and the information that males use to decide among females (Edward & Chapman, 2011).

**Chemical signals**

My research focus is on chemical communication, which is used by a wide range of animals (Wyatt, 2003) and plants (Adler, 2011). Since a variety of types of information can be transmitted about individuals (e.g., species, sex, age, sexual receptivity, individual status and body condition) and locations (e.g., features of oviposition sites or food resources, Wyatt, 2003), both static and dynamic signal components are expected. Arthropods commonly use semiochemicals, which are chemicals that carry information that allows recognition or localization of a potential mate, prey, oviposition site or food resource (Leal, 2005). Sex pheromones are semiochemicals that attract the opposite sex of the same species during the reproductive season, which elicit searching and courtship behavior, and can synchronize the reproductive activity of potential mates in space and time (Karlson & Luscher, 1959; Wyatt, 2003). The first isolated and chemically characterized (structure and atomic composition) sex pheromone was the pheromone produced by females of the silkworm *Bombyx mori* (Lepidoptera, Bombycidae), which attracts conspecific males (Karlson & Butenandt, 1959). Since then, more than 3000 chemical components that affect sexual behavior of males and females have been identified (Symond & Elgar, 2008). The composition and the components of a sex pheromone are typically species specific, resulting in a huge diversity of pheromones in nature (Symonds & Elgar, 2008; Wyatt, 2003). Most of these have been identified in insects, particularly in pest species (Symonds & Elgar, 2008; Wyatt, 2003), and an extensive database of insect semiochemicals exists ([http://www.pherobase.com/](http://www.pherobase.com/)), many of which form
the basis for pheromone traps used in pest control. The available data have suggested two main patterns of diversification of pheromones exist across related (insect) taxa: (1) gradual change accumulates slowly over time through neutral selection or drift such that more closely related taxa show considerable overlap in pheromone composition, with changes in relatively few components of the signal with each lineage divergence (speciation event) and (2) large shifts in the composition of pheromones through natural or sexual selection such that the most closely related taxa show very few chemical similarities, with the frequent appearance of novel pheromones accompanying species divergence (Symonds & Elgar, 2008).

Overlapping variation in pheromone composition between related species arises when diversification is accompanied by the loss or gain of single components in the pheromone, or a change in the proportions of components (Roelofs & Brown, 1982). As a result, closely related species could have similar, or even identical, pheromone compositions. This pattern has been argued to be most common in pheromones used in non-sexual contexts (e.g. pheromones of aggregation, Symonds & Wertheim, 2005; Symonds & Elgar, 2008), where selection for differentiation of pheromone production or reception may be minimal, but genetic drift can lead to the gradual accumulation of differences. For example, different species of ants from the genus *Pogonomyrmex* use the same components, but with different proportions, to indicate food localization (Holldobler et al., 2001). Similarly, *Drosophila* species show gradual variation in aggregation pheromones, with closely-related species more similar in their pheromone chemical components than distantly-related species (Symonds & Wertheim, 2005).
The second pattern involves substantial changes in pheromone composition, where diversification is coincident with the evolution of new pheromones that are completely different from the antecedents, through significant shifts in pheromone components (Symonds & Elgar, 2008). This results in the most closely related species having very different pheromones. This process is expected to have played a major role in the evolution of sex pheromones, due to strong selection on senders and receivers for avoiding wasted effort in heterospecific matings. Substantial divergence in sex pheromone composition would mean that sibling species could largely avoid costly mating mistakes during sexual encounters (Symonds & Elgar, 2008). For example, in bark beetles (Dendroctonus and Ips), aggregation pheromones are also used as sex pheromones, and closely-related species are more different in their chemical components than expected by chance (i.e., as would be expected due to genetic drift, Symonds & Elgar, 2004). Selection for diversification of sexual signals may be particularly strong in bark beetles because there are apparently few other (pre-mating or post-mating) blocks to hybridization (Symonds & Elgar, 2004).

In contrast, even if pheromones are used in sexual contexts, selection for divergence in composition may be absent or minimal if there are other pre-mating or post-mating blocks to hybridization, or if the probability of encountering heterospecific congeners is low. For example, species that utilize other modes of communication in coordinated signals (i.e., multi-modal signals, Costa & Capocasale, 1984; Costa-Smith & Machado, 2012; Gonzalez et al., 2015; Hebets & Rundus, 2011; Stratton & Uetz, 1983) may diversify in other modalities and thus experience little selection on pheromones. Species that are genetically isolated from congeners because they are allopatric, allochronic or utilize different ecological niches may also show minimal differences in sex pheromones (Higgle et al., 2000). However, in
Tephritid flies of the genus *Bactrocera*, in which there are other mechanisms for avoiding hybridization (e.g. great ecological diversity), and there is a positive correlation between pheromone differences and genetic divergence suggesting a gradual change, there are also large differences in sex pheromones between closely-related species. This may suggest that large changes in pheromone composition could be achieved even when there are other modalities to promote reproductive isolation (Symonds et al., 2009).

**Sex pheromones: mate attraction, localization, and courtship**

Species-specific mate attraction as a result of sex pheromones produced by males and females has been well-documented in several taxa (Ache & Young, 2005; reviewed by Smadja & Butlin, 2009). For example, female *Xiphophorus nigrensis* (swordtail fish) using olfactory cues are more attracted to the cues from conspecific males than to those from heterospecific *X. cortesi* or *X. montezumae* (McLennan & Ryan, 1999). Furthermore, in two sibling species of amphibious sea snakes (*Laticauda colubrina* and *L. frontalis*), males directed courtship behavior to pheromone from conspecific but not heterospecific females (Shine et al., 2002). In most invertebrate taxa, females are in general the sex that produces pheromones for mate attraction/localization, and these are airborne chemical blends which use air flow to transmit the signal (Bailey, 1991; Gaskett, 2007; Holwell et al., 2007; Johansson & Jones, 2007; Wyatt, 2003). Males from several arthropods species are able to detect females from long distances using airborne pheromones (Gaskett, 2007; Johansson & Jones, 2007; Wyatt, 2003). For example in the aphid *Phorodon humuli* males are able to locate female’s airborne pheromones from a distance of 2 to 6 m (Hardie et al., 1996). Moreover, males will use the information encoded in the airborne pheromones not only to
locate conspecific females but also to discriminate them from heterospecific females (Hardie et al., 1990, 1992, 1994; Johansson & Jones, 2007; Lofstedt et al., 1991).

In addition to facilitating attraction, sex pheromones can also trigger the initiation of courtship behaviour. This function often works at close range after a potential mate is localized, and involves direct contact between male and female (and their semiochemicals) or areas or substances associated with the opposite sex (e.g., burrows, silk). Response to such contact pheromones is another important opportunity for discrimination as courtship may only proceed when in the presence of a conspecific with particular traits, as signaled in contact pheromones, or courtship may occur, but with reduced effort that is less likely to result in copulation (Gaskett, 2007; Johansson & Jones, 2007; Thomas, 2011; Wyatt, 2003).

**Sex pheromones & mate choice**

Chemical information, particularly as found in sex pheromones, is not only critical for finding mates, but can also provide males with the information needed to decide how to balance the costs and benefits of attempting to mate with or forgoing a detected female. This decision should rest on three factors; the expected reproductive output from the current female, how mating or a mating attempt would affect the probability of future matings, and the expected reproductive benefit derived from other likely mates. This will involve assessment of whether the female is a conspecific, but also finer details of female phenotype.

Dynamic features of sex pheromones can provide information about reproductive or developmental status. For example, males of some species use these substances to detect and recognize females that will soon moult to adulthood, which allows them to attempt to mate
with recently-matured females (e.g. Hardege & Terschak, 2011; Jackson, 1986a). Copulating with new adults before another male is crucial in mating systems where females are monogamous or there is a strong sperm priority for the first male (Fernández-Montraveta & Cuadrado, 2003; Johansson & Jones, 2007; Thomas, 2011). Furthermore, some females, once they are mated, either stop producing sex pheromones, produce lower levels, or produce new components of the signal that may indicate that they are not receptive to additional copulations (Ayasse et al., 1999; Baruffaldi et al., 2010; Carriere & McNeil, 1990; Simmons et al., 2003; Stolz et al., 2007; Trabalon et al., 1996). Sex pheromones can also provide information to males about female condition and hunger. For example, female moths fed only water are less attractive to males because they do not produce the same components in their sex pheromones as do females fed sugar (Harari et al., 2011), suggesting a direct link between nutrition and signal structure. Male mantids are also less attracted to the sex pheromones produced by hungry females (Lelito & Brown, 2006, 2008; Liske & Davis, 1987) although in this case it is not clear whether the pheromone changes in terms of quality or quantity.

Females, on the other hand, may compete with other females to attract males by producing a stronger signal or by starting to call males sooner when they are surrounded by other females (Harari et al., 2011). Females who are close to starvation may even signal receptivity deceptively; putting all their energy in one last effort to attract males and eat them (Barry, 2015). Under this scenario the use and manipulation of the information by females and males provides information about the mating system and the reproductive strategies of each sex, which can inform studies of the mechanism, function and evolution of the signals,
Chemical signals in spiders

In my thesis I focus on signal diversity in sex pheromones among web-building spiders. In 1968, Rovner was the first to use the term ‘sex pheromone’ for a spider, the wolf spider *Rabidosa rabida*. Since then several studies have been explored the use of chemical signals in spiders (reviewed in Gaskett, 2007; Schulz, 2013), and the first spider pheromone was characterized by Schulz and Toft in 1993 (the sex pheromone of *Linyphia triangularis* females). This was almost 34 years after the first pheromones were characterized in insects, and since then, only 8 female sex pheromones have been characterized in spiders, each from a different genus, making comparative study impossible. The huge difference in our knowledge of insect compared to spider pheromones could be due a number of factors. In addition to this being a relatively young field, spiders apparently produce a relatively small amount of pheromones, many pheromones must be isolated from silk, effective bioassay methods for spiders were only developed fairly recently, it was (and is) unclear where pheromone production occurs, (Gaskett, 2007; Schulz, 2004, 2013) and, unlike insects, there are relatively few commercial applications for synthesized spider pheromones. Nevertheless, our current knowledge suggests that web-building spiders frequently use chemical signals for finding a mate and triggering courtship (Foelix, 2011) and females are, in general, the sex that releases cuticular and/or silk-based sex pheromones to attract males, which can be airborne or received via contact with chemoreceptors on the male’s pedipalps (Foelix, 2011; Gaskett, 2007). Airborne pheromones primarily attract males and elicit male searching.
behaviour, while contact pheromones stimulate male courtship behavior (Herberstein, 2011; Gaskett, 2007).

**Inter-specific patterns**

In many spiders, roving males must locate females, and natural and sexual selection to minimize searching costs may lead to the evolution of male’s ability to discriminate chemical cues from heterospecifics relative to conspecifics. In wolf spiders (Lycosidae) and wandering spiders (Ctenidae), males respond to pheromones on silk threads left behind by mobile females, and in some cases may encounter heterospecific congeners as well as conspecifics when searching for mates. Robert and Uetz (2004) exposed males of the wolf spider *Schizocosa ocreata* to silk threads produced by heterospecific females, and showed that *S. ocreata* males responded with lower courtship intensity to pheromones of distantly related congeners compared to conspecifics, but that courtship intensity remained high in response to pheromones of closely related congeners. These findings are consistent with the idea that female chemical signals could be conserved between closely related species, and may diverge gradually according to phylogenetic distance. Similarly, among wandering spiders of the genus *Cupiennius*, males of two sympatric species and one allopatric species respond most strongly to conspecific females, but also respond to the pheromone-laden silk of heterospecific females (Barth & Schmitt, 1991). Males of the two sympatric species reacted more strongly to heterospecific females of the sympatric rather than the allopatric species, whereas males of the allopatric species showed lower responses to heterospecifics on average. In these species, however, interspecific copulation is rare. Despite male responses to pheromones, sexual interactions fail at various points in a multi-step courtship which
involves vibratory and tactile components as well as female responses. Although the phylogenetic relationship among these species remains unknown (Barth & Schmitt, 1991), there is clearly no evidence for character displacement in pheromones or male responses. Moreover, this example suggests that even among sympatric species, the existence of complex, multi-modal courtship could reduce selection for species discrimination based solely on pheromones (Gaskett, 2007; Schulz, 2004, 2013).

Relatively few studies in spiders have looked into heterospecific and conspecific discrimination by males (reviewed in Gaskett, 2007; Schulz, 2004, 2013). While a handful of studies focus on web-building spiders, most have been performed on spiders that are active hunters in which both sexes are nomadic and other channels of communication like vision or vibration could play a key role in mate discrimination. A review of the literature in this area shows evidence both for and against successful discrimination of conspecifics, but since these studies were not considered in a phylogenetic context, it is not possible to draw general conclusions about causes of these differences (e.g., males able to discriminate: Agelenidae: Roland, 1984; Trabalon et al., 1996; Salticidae: Cerveira & Jackson, 2013; Cross & Jackson 2013; Nelson et al., 2012; Willy & Jackson, 1993; Linyphiidae: Schulz and Toft, 1993, males not able to discriminate Lycosidae: Costa & Capocasale, 1984; Robert & Uetz, 2004 a,b; Stratton & Uetz 1983; Araneidae: Olive, 1982; Threchaleidae: Costa-Smith & Machado, 2012) . For example Nelson, Warui & Jackson (2012) tested the ability of males to discriminate conspecifics using seven species from two sub families of Salticidae, but the evolutionary relationships among these species was (and is) unclear. In the wolf spiders *Lycosa thorelli* and *L. carbonelli* males exhibit courtship displays when in contact with the silk of heterospecific females, but seismic (vibratory) and visual cues from females and
males eliminate the risk of hybridization, even though are able to produce viable offspring in laboratory conditions (Costa & Capocasale, 1984). Overall, understanding processes influencing the evolution of male discrimination and/or females’ chemicals cues (and other communication modes), is rudimentary in spiders (reviewed in Gaskett, 2007; Schulz, 2004, 2013). Comparative studies are essential to advance this field, but this is usually not possible because there are not many taxa with well-supported species-level phylogenies in which experimental studies are also tractable.

**Intra-specific patterns**

Males that are seeking mates may also discriminate among females on the basis of factors that will affect their overall reproductive fitness (Bonduriansky, 2001). Intra-specific discrimination among potential mates by male spiders appears to focus primarily on female developmental stage and mating history, age, and recent diet (‘hunger’ level). These factors may affect the expected reproductive output of potential mates as well as the likelihood of mating successfully. The ability of male spiders to discriminate between virgin (unmated) and mated females using chemical cues has been reported from field and laboratory studies across families (Gaskett, 2007; Salticidae: Jackson, 1981, 1986a; Theridiidae: Anava & Lubin, 1993, Andrade & Kasumovic, 2005; Stolz et al., 2007; Lycosidae: Baruffaldi & Costa, 2010, 2014; Roberts & Uetz, 2005; Rypstra et al., 2003). In general, virgin females are preferred by males in species with first male sperm priority (the sperm of the first male to inseminate the female is used first for fertilization, Elgar, 1998) and/or species where the female’s post-mating sexual receptivity is low (Foelix, 2011; Herberstein, 2011; Huber, 2005; Snow & Andrade, 2004). For example, relative to their behaviour when they detect
unmated females, males exposed to silk from mated females may decrease the intensity and frequency of courtship (Lycosidae: Baruffaldi & Costa, 2010, 2014; Roberts & Uetz, 2005; Rypstra et al., 2003). Responses to unmated females occur primarily after they are sexually mature (Gaskett, 2007), although males of some species are attracted to last-instar immature females. The ability of males to recognize female reproductive maturity (e.g. immature or adult) has important implications for male mating decisions. In some species immature females are sedentary and males use pheromones to locate these females and guard them until they reach adulthood and are able to mate (Fernandez-Montraveta & Cuadrado, 2003; Jackson, 1986a,b). Once they locate immature females or recently moulted virgin females, males could copulate with post-moult females when they still have a soft exoskeleton (= teneral females) and thus avoid predation risk since females are largely defenseless of this time (Gaskett, 2007; Herberstein, 2011; Huber, 2005). This may also constitute coercive mating since recently-moulted females are unable to repel less-preferred males (Huber, 2005), although it is not clear whether this behavior is costly to females.

In contrast to males that mate teneral females, in other species males show very little courtship behaviour towards recently moulted adult females (young unmated females), although the frequency and intensity of courtship displays increases as these females age (Baruffaldi & Costa, 2010, 2014; Riechert & Singer, 1995; Roberts & Uetz, 2005). For example, some males show a preference for sex pheromones produced by older virgin females which may be in better condition and more receptive to mating (Baruffaldi & Costa, 2010, 2014; Roberts & Uetz, 2005). In the desert spider Agelenopsis aperta, males show more courtship behavior towards older (rather than younger) virgin females, likely because young females have a smaller chance of survival in desert conditions (Riechert & Singer,
A similar preference by males for older adult females in wolf spiders *Schizocosa malitiosa*, is related to the amount of chemicals that the females produce (Baruffaldi & Costa, 2010, 2014; Baruffaldi et al., 2010), with silk extracts from older adult virgin females having a higher concentration of chemicals than those from young virgin or mated females (Baruffaldi et al., 2010).

In addition to seeking females with high expected reproductive value, males may also seek to avoid pairings that are likely to be costly (as when avoiding heterospecific females). In many spiders, one of the most salient risks for males approaching a conspecific female is sexual cannibalism (where females attack and consume part or all of a male during courtship or mating). In web-building spiders, in general, females are easily able to subdue or capture males since they are often much bigger (sometimes this is extreme, with males dwarfed by females many hundred times their body weight, Foelix, 2011; Huber, 2005), and the male approaches the female on her own web. Moreover, females are frequently under nutrient stress in nature (Stoltz et al., 2010; Wise, 1979), which limits the development and production of eggs. Therefore, in some situations, for example when males are abundant but other prey are not, females will benefit more from eating a male than mating with him (Elgar & Nash, 1988; Johnson, 2001; Roggenbuck et al., 2011; Wilder & Rypstra, 2008). By consuming males, females could increase body mass (Elgar & Nash, 1988; Elgar, 1998), fecundity (Johnson, 2001, 2005) or offspring survival (Welke & Schneider, 2012; Wu et al., 2013). Thus, males need to be very careful and choosy when approaching a female (Huber, 2005). The timing of sexual cannibalism will have important implications for male and female mating decisions (Elgar & Schneider, 2004; Herberstein, 2011). When cannibalistic females typically attack males that are courting (‘precopulatory sexual cannibalism’, Elgar,
1992, 1998; Elgar & Schneider, 2004; Herberstein et al., 2002; Huber, 2005), selection to detect and avoid these females should be strong (e.g. Johnson et al., 2011). If cannibalism occurs during or after mating (‘post-copulatory cannibalism’), cannibalized males may transfer sperm successfully and can still accrue paternity. Nevertheless, if mating with multiple females typically increases male success (Bateman, 1948), males will typically seek to avoid post-copulatory cannibalism. In the polygynous wolf spider *Schizocosa ocreata*, males exposed to female’s silk exhibit more courtship if the female had previously cannibalized a male (and thus was likely satiated) than if the female was starved (Moskalik & Uetz, 2011). Moreover, *S. ocreata* males use chemical cues to recognize and avoid young virgin females because they tend to be more cannibalistic than older females (Robert & Uetz, 2004). Similarly, males of the polygynous western black widow *L. hesperus* reduce courtship effort when exposed to the silk of hungry compared to well-fed females (Johnson et al., 2011), and are less likely to be attracted to the webs of hungry females in the field (MacLeod & Andrade, 2014). Thus, many of the variables that may be revealed in pheromones and suggest variation in female reproductive value can also reveal the risk of sexual cannibalism in different species. Examples include female reproductive status (e.g mated females more likely to cannibalize males), age (e.g. younger females more likely to cannibalize males) and female condition (hungry females more likely to cannibalize males).

In contrast to pre-copulatory cannibalism, if cannibalism occurs during or after mating (post-copulatory cannibalism), cannibalized males may transfer sperm successfully, can still accrue paternity, and thus may not attempt to avoid these matings. In some of these species, cannibalized males actually benefit from through parental investment or mating effort (Elgar & Schneider, 2004; Parker, 1979; Welke & Schneider, 2012). For example,
cannibalized males have higher paternity than males that are not cannibalized in at least two species (e.g. *Latrodectus*: Andrade, 1996; *Argiope*: Schneider et al., 2006), and rather than avoiding cannibalism, males in fact offer themselves to females during mating (somersault behavior of *L. hasselti*: Andrade, 1998, 1996; Forster, 1992). Even with post-copulatory cannibalism, however, if mating with multiple females typically increases male success (Bateman, 1948), males may still seek to avoid sexual cannibalism, although this may be conditional and depend on the balance between the expected reproductive output of a cannibalistic mate compared to expectation of future reproduction with non-cannibalistic potential mates (e.g., Fromhage & Schneider, 2012).

**Potential impact of comparative study of spider pheromone function**

Currently, most of the studies proposing links between signal function and evolutionary history (e.g., degree of sympatry) are based on pest insects (Symonds & Elgar, 2008). This is problematic for (at least) two reasons. First, many of these insects are plant pests, and since there are demonstrated links between host-plant and pheromone composition (Symonds & Elgar, 2004), this ecological factor may be driving diversification independent of the reproductive consequences of pheromone discrimination. Second, this is a very limited taxonomic sample despite the widespread nature of chemical communication, so more studies examining the patterns of pheromone evolution in different taxa are needed (Symonds & Elgar, 2008). Clearly, sex pheromones of spiders can be very information-rich, and are a critical component of successful reproduction, and so this group may yield valuable insights into signal evolution (reviewed in Gaskett, 2007; Schulz, 2004, 2013). However, making inferences about patterns of evolution in spiders is challenging because most past studies use
web or silk rather than isolated chemicals to test communication (thus allowing the possible transmission of non-chemical information, Gaskett, 2007; Schulz, 2004, 2013). In addition, most of the comparative work has been done in active hunter spiders (jumping spiders, wolf spiders, wandering spiders) without any phylogenetic context (see, Robert & Uetz, 2004a, for exception). Web building spiders, given their very different ecology and the potential for the web as a sustained source of signal (compared to the more transient drag lines of wandering hunters), are key candidates for studying pheromone diversity and how diversity could be affected by pheromone function (e.g., attraction [airborne], courtship [contact]). The broad goal of my thesis is to significantly increase our understanding of the processes and underlying sex pheromone diversification and functional flexibility by combining a comparative study of responses to isolated chemicals with an examination of behavioral dynamics related to discrimination and individual fitness in ecologically relevant contexts.

**Thesis goals**

The specific aims of my PhD thesis are to (1) test hypotheses regarding divergence and diversity in pheromone structure and function in focal species of the spider genus *Latrodectus* (the widows; family Theridiidae), (2) combine these tests with behavioural analyses of communication and mating behaviour to increase understanding of how sex pheromones are utilized in mating and (3) examine how sex pheromones vary with individual mating status and condition within focal species with different ecologies and mating behaviours, and determine how this may affect individual fitness and mating decisions.
Latrodectus Natural History

The spider genus *Latrodectus* Walekenaer, 1805 (Araneae: Theridiidae) includes a group of 31 species (“widow spiders”, World Spider Catalog, 2015) with worldwide distribution including the infamous “black widows” of the southern and western USA (Garb et al., 2004; Condy et al., in preparation). Molecular phylogenetics supports two monophyletic clades within the genus: geometricus (3 species, mostly African) and mactans (including the remaining 28 species). The mactans clade includes three well-supported sub-clades (Condy et al., in preparation) that correspond to three different biogeographical regions (SC/BR: subclades/biogeographical regions) in which member species are found (South America, North America, and Australia-New Zealand, Garb et al., 2004, Condy et al., in preparation, Fig. 1-1).

Ecology and Behaviour

*Latrodectus* spp are cobweb weavers, in which spiderlings disperse by ballooning from their mother’s web after emerging from their egg sac (e.g. Suter, 1991), leading to a patchy distribution in the field (Andrade & Banta, 2002; Macleod, 2014; Salomon, 2007). Species in this genus show extreme female-biased sexual size dimorphism, as females have approximately 2 more development instars than males (Forster & Kingsford, 1983; Kaston, 1970; Smithers, 1944), and are several hundred times the male’s body mass at adulthood. Females are mainly sedentary, generally staying on the same web throughout their adult lives (Salomon, 2007). Males mature faster than females, then abandon their juvenile webs searching for females, when they are exposed to a high risk of mate searching mortality.
(Andrade, 2003; Segev et al., 2003; Segoli et al., 2006). In the laboratory, females can survive for 24 months whereas adult males survive a maximum of 2 months (Andrade, 1996; Forster, 1984).

Most studies of widow spiders have concerned their neurotoxic venom, silk production, and distribution (Garb & Hayashi, 2013; Garb et al., 2004). There are also studies of behaviour and ecology in some species, and these suggest extensive interspecific variation in habitat utilization (e.g., plant-host specific species, species that live in burrows, under stones or wood, species that live in forests or on beaches, species that are anthropophillic and invasive and those that are not), degree of sympatry (e.g., species can be found in sympatry [syntopy or allotropy] or allopatry with congeners) and reproductive strategies (e.g., species with and without sexual cannibalism, monogamous or polygamous males and females) (Andrade, 1996; Breene & Sweet, 1985; Garb et al., 2003; Forster, 1992; Kaston, 1970; Knoflach & van Harten, 2002; MacLeod, 2014; Ross & Smith, 1979; Segoli et al., 2006; Segev et al., 2003). In contrast, there is relatively little interspecific variation in morphology and color in the genus; although genital morphology is often a key taxonomic trait in other spider taxa (Levi, 1959, 1983). This may suggest a pattern of relatively rapid speciation (Breene & Sweet, 1985; Forster, 1992; Kaston, 1970; Levi, 1959, 1983; Neumann & Schneider, 2011; Ross & Smith, 1979), which may have been coincident with the evolution of the component of their venom that is highly toxic to vertebrates (Garb et al., 2013).

Mate localization and the initiation of searching behaviour are triggered by male detection of airborne chemicals (sex pheromones) released from the webs or body of females
(e.g. Anava & Lubin, 1993; Andrade & Kasumovic, 2005; Kasumovic & Andrade, 2004; Ross & Smith, 1979). Courtship commences once males touch the female’s silk (Anava & Lubin, 1993; Kaston, 1970) and involves the production of vibrational signals on the female’s web and abdomen. The mating behaviour of several species of Latrodectus has been described (e.g. L. hasselti, Foster 1992; L. hesperus, Herms et al., 1935, Ross & Smith 1979; L. mactans, Breene & Sweet 1985; L. reviviensis, Anava & Lubin, 1993; L. pallidus, Segoli et al., 2006; L. geometricus, Segoli et al., 2008; L. tredecimguttatus, Neumann & Schneider, 2011). The sequence of courtship behaviours by the male is very similar in all these species and includes the production of vibrations, cutting of the female’s silk with addition of the male’s own silk, standing on female, drumming on the female’s abdomen and genitalia, and throwing of silk on or around the female. The duration of courtship behaviour is variable among species and ranges between approximately 10 minutes (L. mirabilis, Baruffaldi unpublished) up to an average of 5 hours (L. hasselti) (Forster, 1992).

During courtship, females rest, ventral surface up on the snare of the web. After courtship the male climbs onto the female’s abdomen with the ventral surfaces in close proximity and the cephalothorax facing in the same direct. Males then insert one of their paired intromittent organs (pedipalps: organs than carry and transfer sperm) into one of the female’s genital openings. Adult females’ external genitalia are a hardened, raised area of cuticle (epigynum) within which are two genital openings, each of which is connected to one of two sperm storage organs (spermathecae) from which sperm are taken in roughly equal proportions at fertilization (Foelix, 2011; Snow & Andrade, 2005). To maximize paternity, males must inseminate both spermathecae, and this is accomplished only if they copulate twice, inserting one of their 2 pedipalps at each copulation (one ‘complete’ mating). The two
palpal insertions are in general separated by a period of additional courtship on the web (~20 min. Forster, 1995). Males frequently leave behind a sperm plug (the broken tip of their copulatory organ), which does not necessarily prevent male or female remating, but is linked to first-male sperm precedence if they deposit the plug successfully (Macleod, 2014; Neumann & Schneider, 2011; Snow & Andrade, 2005; Snow et al., 2006).

Even though the initial copulatory position is very similar across species (e.g. males insert one palp at a time while standing on female’s abdomen), male behavior during copulation can be very different. Some males will flip their abdomen onto female’s fangs during mating (somersault behavior) and females will eat them (e.g., *L. hasselti*, Foster, 1995; *L. geometricus*, Segoli et al., 2008), whereas in others, despite female attacks, males may copulate and escape from the female (e.g. *L. hesperus*, *L. mactans*, *L. variolus*). Despite some differences such as these, Kaston (1970) observed males of some species from the North-American sub-clade court and mate with heterospecific females, and Ross & Smith (1979) showed such heterospecific courtship may be initiated by exposure to silk alone, suggesting a conservation of pheromones or other cues. Furthermore, Schmidt (1991) showed that in some situations heterospecific matings were able to produce hybrids.

**Chemical communication in Latrodectus**

This genus may be an interesting model for understanding the evolution of signal diversity for a number of reasons, which I summarize here and expand on below. First, signaling can be studied in the laboratory and field (Kasumovic & Andrade, 2004; MacLeod & Andrade, 2014). Second, their contact pheromones have been chemically characterized (Jerhot et al., 2010; Scott et al., 2015; Kiefer, Baruffaldi, Andrade, Schulz in progress). Third past work
suggests some pheromone components are conserved across species (Kasumovic & Andrade, 2004; Ross & Smith, 1979; Schmidt, 1991), but others may be species or population-specific (Kasumovic & Andrade, 2004), suggesting an interesting diversity of signals. Moreover, although there is limited current evidence for syntopy, there are some species that are likely to have experienced sympatry relatively recently—for example, the species in the mactans clade that are found in the USA, including three focal species examined here (L. mactans, L. hesperus and L. various, Fig. 1-1). Finally, the genus Latrodectus is one of few spider genera in which there is information about the phylogenetic relationships among species, satisfying an important requirement for testing predictions about the evolution and diversity of chemical signals.

At the level of intra-specific variation, Latrodectus spiders may also allow key investigations into plasticity in chemical communication, the importance of sex pheromones for localizing high quality conspecific females, but also for employing the best reproductive strategy according to the information received (e.g., Kasumovic & Andrade, 2006).

**Intra-specific variation**

Similar to other spiders, chemical communication has a very important role in mating decisions in black widows. Males of *L. hasselti* are able to discriminate between juveniles and females with different reproductive status (unmated and mated) using contact pheromones only (demonstrated with silk extracts, Stoltz et al., 2007). Moreover, *L. hesperus* males use airborne pheromones produced by the female and/or her web to discriminate between unmated and mated females, being more attracted to unmated females’ cages in the field (MacLeod & Andrade, 2014). In addition, in *L hasselti* males exhibit more mate
searching on silk extracts from unmated than recently mated females (Stolz et al., 2007), moreover mated females producing the chemical responsible for elicit male courting behavior (Jerhot et al., 2010). However, mated females seem to starts pheromone production again months later when they have used sperm or just before overwintering (Perampaladas et al., 2008), showing female pheromone production may be strategic, may reflect receptivity, and will set limits on when males can mate. Experiments also suggest that chemicals and/or other cues associated with silk may allow L. hesperus males to discriminate female feeding history (Johnson et al., 2011; MacLeod & Andrade, 2014). Detection of pheromones produced by virgin females also triggers developmental shifts in L. hasselti males. Immature males that develop while surrounded by airborne pheromone of females developed faster than those develop without this environmental cue, and this difference could potentially affect their ability to reach females before competitors (Kasumovic & Andrade, 2006).

**Inter-specific variation**

Field work with males of L. hesperus in British Columbia (Vancouver) suggests there may be similar components in the airborne pheromones of the allopatric, distantly related species L. hesperus and L. hasselti (Fig 1, Kasumovic & Andrade, 2004). The British Columbia population of L. hesperus males were attracted to airborne pheromones produced by L. hasselti females in addition to syntopic conspecific females. However, males were much less attracted to conspecifics from allopatric populations of L. hesperus (Arizona), suggesting populations of L. hesperus may be diverging (Barrett & Hebert, 2005; Kasumovic & Andrade, 2004).
Focal species

In my thesis I studied 6 representative species from the *Latrodectus* genus (Fig. 1-1). For the comparative analysis in my second chapter, these were chosen to represent the two major clades from the genus: *geometricus* clade (*L. geometricus*) and *mactans* clade, and also 3 well-supported SC/BR from within the *mactans* clade: South America (*L. mirabilis*), North America (*L. hesperus, L. mactans, L. variolus*) and Australia- New Zealand (*L. hasselti*). I included 3 species from inside the North American SC/BR to allow comparisons of closely related species in addition to the more distantly related species from other clades or SC/BR. One of these species was the focus of an intra-specific study of female fitness and pheromone signaling tactics in chapter four (*L. hasselti*). In chapter three I used species from two SC/BR (*L. hasselti* and *L. hesperus*) to make inferences about whether mechanistic links between pheromone production and diet might be common in *Latrodectus* and whether divergent mating systems might predict differences in pheromone-mediated male mate choice. Below I provide some natural history information on each of these focal species and then provide a more detailed overview of the contents of each chapter.

*Latrodectus geometricus* (Koch, 1841). Originally an African species, *L. geometricus* now has a worldwide distribution, and is the only species in my thesis representing the *geometricus* clade (which includes only 3 species). The expanded distribution of this species seems to have started at some point during the 1800’s, when records of *L. geometricus* in South America appeared (Garb et al., 2004). Even though *L. geometricus* has a broad distribution across all continents (except Antarctica), and is now sympatric with species from the *mactans* clade in several locations, there are no indications or records of hybridization
with other *Latrodectus* species (Garb et al., 2004). This species has extreme female-biased sexual size dimorphism (mass of: adult females ~ 201.59 mg; adult males ~ 2.86 mg, size ratio: 70.5, Baruffaldi unpublished; Segoli et al., 2008). Many males perform the somersault behavior during mating with adult females (Segoli et al., 2008), and so males are often monogynous (mate only once with an adult female).

**L. hasselti** (Thorell, 1870). Originally from Australia, *L. hasselti* was recently introduced to New Zealand (Forster & Kingsford, 1983) and Japan (Ori, 1996), and is the only species in this thesis from the Australia-New Zealand SC/BR (which includes 2 species). *L. hasselti* was introduced in the 80’s to New Zealand (Forster & Kingsford 1983; Forster 1984, 1992; Raven & Gallon, 1987; Vink et al., 2011) and since then hybridization with the endemic sister species *L. katipo* (Powell, 1870) has been reported (Vink et al., 2008). Not only are males of *L. hasselti* attracted to the pheromones produced by *L. katipo* females, but they are also able to mate and produce viable offspring with them (Forster, 1984, 1995). Although males perform the somersault with *L. katipo* females, females do not eat them (Forster, 1992, 1995). However *L. hasselti* females do not mate with *L. katipo* males (Forster, 1992, 1995), showing an example of directional hybridization (Forster, 1984, 1995). A similar situation was observed when males of *L. hasselti* successfully mate with *L. tredecimguttatus* females, generating viable offspring (Schmidt, 1991).

*L. hasselti* has been the backbone of behavioral and ecological studies of *Latrodectus* species. In this species there is also an extreme bias in body size towards females (female mass ~ 204.69 mg, males weight ~ 3.35 mg, size ratio: 61.1, Baruffaldi unpublished) and males also perform the somersault behavior, with males that are eaten by females achieving
more paternity than males that survive (Andrade, 1996, 1998). Moreover, males are able to
discriminate unmated from mated females using sex pheromones (Perampaladas et al., 2008;
Stolz et al., 2007), which is really important in this species in which males deposit a plug (tip
of the male genitalia) into the female genitalia, leading to first male sperm precedence (Snow
& Andrade, 2004).

For female *L. hasselti*, pheromone production starts after sexual maturity, ceases
immediately after mating (Stolz et al., 2007), but starts again 3 month of the first mating
(Perampaladas et al., 2008), showing plasticity in the production of this signal.

*L. mirabilis* (Holmberg, 1876). Originally from South America, little is known about the
distribution, sexual behavior or ecology of this species (Berrantes & Eberhard, 2010; Garb et
al., 2004; Gonzalez, et al., 1998). Most of the studies have been focused on morphology in
this size-dimorphic species (female mass ~ 176.10 mg, male mass ~ 2.83 mg, size ratio: 62.2,
Baruffaldi unpublished), venom and prey capture (Cunningham et al., 2000; Pompozzi et al.,
2013). *L. mirabilis* is the only species from the South American SC/BR (which includes
approximately 5 species) used in this thesis.

*L. hesperus* (Chamberlin & Ivie, 1935). Native to North America, this species is distributed
throughout western North America, from central British Columbia and Alberta in the North
through Texas in the south (Kaston, 1970). *L. hesperus* is one of the three species from the
North American SC/BR (total of 4 species) used in this thesis. *L. hesperus* is currently
sympatric with two other North American species (Kaston, 1970) and *L. geometricus* at
different parts of its range. Moreover, populations of *L. hesperus* from different locations are
apparently reproductively isolated, showing diversification in pheromone-based mate
attraction (Kasumovic & Andrade, 2004) and higher variation at the COI locus than might expected for one species (Barrett & Hebert, 2005). Even though there is still female-biased size dimorphism, it is not as extreme as in the other species (female mass ~ 252.1 mg, male mass ~ 9.67 mg, size ratio: 26.1, Baruffaldi unpublished). In general males are polygynous (MacLeod, 2014). Sexual cannibalism is generally rare among well-fed females, but when it occurs cannibalism occurs during courtship (pre-copulatory) and prevents males from mating (Johnson et al., 2011).

Previous studies in this species showed that *L. hesperus* males are more attracted to unmated, well-fed females rather than mated or hungry females in the field, this discrimination is via airborne (volatile) pheromones (Macleod & Andrade, 2014), and that male courtship effort also shows this preference in laboratory mating trials (Johnson et al., 2011).

In addition, *L. hesperus* males respond to heterospecific pheromones—they are attracted to *L. hasselti* females’ airborne pheromone in the field (Kasumovic & Andrade, 2004) and exhibit courtship displays when exposed to *L. mactans* webs in the laboratory, suggesting the conservation of sex pheromones between these species (Ross & Smith, 1979). However, when interspecific crosses were conducted between *L. hesperus* and *L. mactans*, only 3 of 27 attempts ended in successful mating but without the development of the egg sacs. On the other hand, in crosses between *L. hesperus* and *L. variolus* there were no matings after 18 attempts (Kaston, 1970). Unfortunately, inter-specific studies do not include systematic information about the progress of courtship and copulation, nor appropriately
controlled assessments of responses to pheromone independent of other cues (e.g., Gaskett, 2007).

**L. mactans** (Fabricius, 1775). Originally from North America, this is a common species in the southeastern United States, and is the second species from the North America sub clade in this thesis. The behavior, ecology and development of this species have been documented by Kaston (1970). In *L. mactans* sexual size dimorphism is similar to that found in *L. hesperus* (female mass ~ 171.1 mg, male mass ~ 5.15 mg, size ratio: 33.2, Baruffaldi unpublished), sexual cannibalism is not observed during mating (Breene & Sweet, 1985; Kaston, 1970), and males are assumed to be polygnous. When 4 interspecific crosses were performed between *L. variolus* and *L. mactans*, no matings occurred (Kaston, 1970).

**L. variolus** (Walckenaer, 1837). Originally from North America, this is a common species in the northern United States of America and adjacent Canadian provinces, although there are records as far south as central Florida, where it is apparently sympatric with (introduced) *L. geometricus* and possibly *L. mactans*. This is the third species from the North America sub clade used in this thesis. The behavior, ecology and development of this species is similar to the other North American widows (Kaston, 1970), but the degree of dimorphism is the lowest of the focal species in this thesis (female weight ~ 180.63 mgr, male weight ~ 11.85, size ratio: 15.24, Baruffaldi unpublished). Little is known about the sexual behavior in this species, however sexual cannibalism may be uncommon (Kaston, 1970).
Overview of Chapters

In my second chapter I examined pheromone functional diversity using a chemical and behavioral perspective. Using the 6 representative *Latrodectus* species described above, I studied divergence in sex pheromone function and male discrimination, including (a) the response of males to silk-based (contact) and airborne pheromone of heterospecific compared to conspecific females, and (b) the likelihood and fitness effects of hybridization with heterospecific females. There is little evidence for historic sympatry being sufficient to select for divergent evolution of sexual signals in this genus. Thus, I tested the hypothesis that sex pheromones have undergone gradual evolution. I hypothesized that sex pheromones would be more divergent in distantly related compared to closely related species, and predicted this would be reflected in male responses to contact pheromones extracted from silk, male attraction to airborne pheromones, and male courtship and mating attempts with heterospecific females. I quantified male responses to conspecific and heterospecific females and their sex pheromones using a bioassay of male activity on extracts of females’ silk (see Jerhot et al., 2010; Stoltz et al., 2007), a T-maze 2-choice system for airborne pheromones, and male courtship progress and mating success when paired with heterospecific or conspecific females in the lab. This approach allowed comparison of the effects of pheromones and other cues as pre-mating blocks to hybridization, as well as inferences about the existence of post-mating blocks (Butlin et al., 2012; Howard, 1993). I compared the relative intensity or outcome of male responses within each species, and finally, assessed male and female responses at each stage of mating interactions across species as a function of genetic distance (COI sequence dissimilarity). This set of studies is one of a handful of
comparative tests of evolutionary divergence of sex pheromones, and is also the most comprehensive comparative analysis of arachnid chemical communication available to date. This work will be the backbone for later comparative analyses of signal evolution and speciation in arachnids.

In the remainder of my thesis I examine intraspecific variation in sex pheromones, test developmental and ecological effects on pheromone production in representative species, and use these data to test predictions about female reproductive tactics (Chapter 3 and 4) and the use of pheromones in male mating decisions (Chapter 3).

In my third chapter I examined possible links between silk production, sex pheromone production and female diet in two closely related species. Here I tested both a mechanistic hypothesis about the underpinnings of honest pheromone signaling of hunger by females, and a functional hypothesis about males’ pheromone-based mate choice. In *L. hesperus*, recent work showed that males avoid hungry females in favour of well-fed females (MacLeod & Andrade, 2014) and reduce courtship effort with hungry females (Johnson et al., 2011). Since poorly-fed females also reduce silk production (Blackledge & Zevenbergen, 2007), and the sex pheromone is present on silk, this suggests a possible mechanistic underpinning to honest signaling of hunger in female spiders. In chapter 3 I first replicate the study of diet effects on silk production in *L. hesperus* and examine whether the link between diet and silk production also exists in *L. hasselti* (from another SC/BR), and then in both species, test how silk volume and diet relates to male searching response to silk extracts from conspecific females. Next I examine how comparing responses of males may give insight into the function of male mate preference. The preference of *L. hesperus* males for well-fed
females reduces the risk of pre-copulatory sexual cannibalism (Johnson et al., 2011). However, these results are also consistent with a preference for more fecund females (e.g., Danielson-Francois et al., 2002; Harari et al., 2011; Johnson et al., 2011; Lelito & Brown, 2008; Wise, 1979). Here, I seek insight into the function of male choice by comparing pheromone-based mate preferences of males in *L. hesperus* (where cannibalism occurs before mating and reduces male fitness, Johnson et al., 2011) and *L. hasselti* (where post-copulatory sexual cannibalism increases fitness, Andrade, 1996). If male choice of well-fed females is primarily a way of avoiding cannibalism, I predicted this preference in *L. hesperus*, but not *L. hasselti*. If, however, male choice is a preference for fecund females, we expected to find the preference in both species. This study was published in *Animal Behaviour* (Baruffaldi & Andrade, 2015) and is reprinted with permission from the journal.

In my fourth chapter, I seek insight into the how female pheromone production is related to the fitness effects of a novel male mating tactic in *L. hasselti*. I quantified the response of females mated to males using different tactics in terms of variation in the females’ response to males’ behavior, post-mating longevity, egg sac development and post-mating pheromone-mediated attraction of alternative mates. *Latrodectus hasselti* males have a surprising mating tactic in which they tear through the exoskeleton of last-instar juvenile females (immature females) and inseminate their recently developed sperm storage organs (immature mating, Biaggio, 2007). The behaviour is apparently adaptive for males and occurs in nature (MCBA personal communication, but consequences for females are unclear (Biaggio, 2007). Immature-mated females rarely show typical mate choice because males do not court them, but produce spiderlings as adults after a normal moult. Here I predicted that, if immature-mating maladaptively circumvents mate choice of *L. hasselti* females, immature-
mated females may resist mating, produce fewer eggs and show reduced longevity. Moreover, if immature-mating is maladaptive, I predict females will continue producing sex pheromones as adults to solicit additional matings given the lack of choice of the first mate. In contrast, I predict females will cease pheromone production as adults if immature-mating is adaptive or neutral. Identifying the effect of immature-mating on female fitness and female response in terms of mate attraction is important as the tactic may affect the direction and intensity of sexual selection in nature, and this tactic may be common across the genus (also see Appendix 2).
References


Jackson, R.R. (1986a). Use of pheromones by males of *Phidippus johnsoni* (Aranea, Salticidae) to detect subadult females that are about to molt. *Journal of Arachnology*, 14, 137-139.


Figure 1. Phylogeny of 23 of the 31 *Latrodectus* species recovered from a Bayesian analysis of a 505bp of CO1 gene sequence analysis modified from (Condy et al., in preparation). Focal species for these studies are highlighted with boxes and include one representative from the *geometricus* clade (brown), and 5 from the *mactans* clade, distributed across three representative sub-clades that correspond to distinct biogeographical regions SC/BR (South American, purple; North American, black, Australia-New Zealand, red). Bayesian posterior probabilities are shown above each node with the well-supported nodes for the two clades and focal SC/BR within the *mactans* clade highlighted (*). The topology of this phylogeny with respect to the focal species is qualitatively similar to that published previously based on a smaller taxonomic sample (Garb et al., 2004), and to that derived from a preliminary multi-gene analysis (Condy et al., in prep).
Figure 1-1

A phylogenetic tree showing the relationships among different species of L. geometricus and their clades. The tree highlights species found in Australia-New Zealand, North America, and South America. The tree includes branches with support values and labels for specific species. The tree is labeled with different colors to distinguish among different clades: geometricus clade, mactans clade, Australia-New Zealand, North America, and South America.
Chapter 2

Phylogenetic pattern of sex pheromone discrimination in spiders: evidence from widow spiders

Abstract

Understanding of signal diversification predicts sympatry will favour significant divergence in structure or response between closely related species, whereas signals that do not affect the risk of interbreeding will slowly accumulate differences over evolutionary time. Here I studied functional divergence in sex pheromones focusing on six species of widow spiders (Thereiidae: *Latrodectus*: *L. geometricus*, *L. mirabilis*, *L. hasselti*, *L. hesperus*, *L. mactans* and *L. variolus*) representing different clades and biogeographic regions. I test the hypothesis that, in the absence of a recent history of sympatry, divergence in sex pheromones and mate recognition will be best predicted by phylogenetic distance in *Latrodectus* spiders. Among spiders, females release cuticular and/or silk-based sex pheromones that can be detected as airborne volatiles (attracting males) or as contact chemicals (initiating courtship). I quantified male responses to conspecific and heterospecific females and their sex pheromones using a bioassay of male searching activity on methanol extracts of females’ silk, a T-maze choice apparatus with airborne pheromones, and male courtship and mating when on the webs of females. I compare male responses in light of the phylogenetic topology of the genus, and as a function of estimated genetic distance between species (COI sequence dissimilarity). I show that, consistent with our hypothesis (1) male *L. geometricus*, the most distantly related
of my focal species, respond only to silk extracts from conspecifics; whereas males from the other species also responded to extracts from more closely related heterospecific species and (2) the same response was observed with airborne pheromones. Although males were able to mate with heterospecifics, and this was common for some species pairs, no viable offspring were produced.
Introduction

Theoretical work predicts that divergence in sexual signals between closely related species could be driven by (at least) three processes. First, if there is a risk of hybridization in sympatry, divergence will be influenced by historic and current distribution of the species and phylogenetic relatedness. Thus the signals of closely related species with a history of sympatry may be more divergent than more distantly related species, with closely related sympatric species having developed signal diversity via selection for pre-mating isolation. Even though these species may experience a reduction in pre-mating isolation after a period of allopatry (in the absence of selection maintaining these mechanisms, Noor, 1996; Servedio, 2004; Wellenreuther et al., 2009), a phylogenetic pattern may still be maintained. Second, differences in signals may accumulate gradually over evolutionary time via genetic drift, particularly if there is no risk of hybridization (e.g. species are in allopatry) or other mechanisms to avoid hybridization exist. Although mating signals within species are normally under strong stabilizing selection (Patterson, 1985), some compounds in a multi-component signals may vary without (initially) affecting the net attractiveness of the signal. Thus, there may be positive links between genetic distance and signal diversity (Bradbury & Vehrencamp, 2011; Butlin et al., 2012; Kirkpatrick & Ryan, 1991; Otte & Endler, 1989; Philips & Johnston, 2009; Robert & Uetz, 2004a; Wilson, 1975). In this case, gradual changes in the signals may arise over evolutionary time and relatively small differences will be observed in closely related species (Coyne & Orr, 2004; Phillips & Johnston, 2009; Robert and Uetz, 2004a).
A third process that may be important is variation in selection in allopatry. In this case, variation in sexual or natural selection on signals in isolated populations can lead to divergence or similarities which are unlikely to be linked with the phylogenetic history of the group. For example, natural selection on how well a signal is transmitted and perceived in a particular type of environment (sensory drive) could alter signals in allopatic population. In some species, similarity or divergence of a sexual signal can be affected by ecological constraints, and equivalent niches or anatomic similarities that could affect how a signal is emitted or received (Boughman, 2002; Harmon et al., 2005; Losos, 1992). Therefore, some species that have similar ecologies may retain similar ancestral components or converge on novel components that allow signal transmission in that environment (Henry et al., 1999; Jones & Holderied, 2007; Tobias & Seddon, 2009). For example, in spiders, web location and structure, sun exposure, humidity and temperature can influence airborne pheromone spread, and perhaps retention of contact pheromone on silk. It has been shown elsewhere that rain and humidity reduces the persistence of the contact pheromone on females silk (Baruffaldi et al., 2010), and also that spiders associated with aquatic habitats have pheromones with different chemical properties than related taxa (Lizotte & Rovner, 1989). It has been well-documented that sexual selection can also lead to unpredictable elaboration of different aspects of signals in allopatric populations (Anderson, 1994; Boughman, 2002; Coyne & Orr, 2004; Huber, 2005; Kirkpatrick & Ryan, 1991; Philips & Johnston, 2009; Simmons & Elgar, 2008). Signals that diversify via sexual selection in allopatry may show little correlation with phylogenetic history, particularly if variation arises through Fisherian processes, or through environmental variation in traits that confer high male fitness.
In this study I examine the evolution of diversity in chemical signals, which are used by a wide range of animals and can transmit a variety of different types of information about individuals (e.g., species, sex, age, sexual receptivity, individual status and body condition) and locations (e.g., features of oviposition sites or food resources, Leal, 2005; Wyatt, 2003). While most the studies of signal diversification have focussed on the sexual signals of males, in the chemical channel females frequently produce signals and most studies focus on the diversity of females’ sexual signals. Moreover, most of what we know about the utilization and structural composition of pheromones has been identified in female insects, particularly in pest species (Symonds & Elgar, 2008; Wyatt, 2003). Here I focus on the evolution of diversity in chemical signals (pheromones) in spiders, in which sex pheromones are primarily produced by females (Schulz, 2004, 2013). Studying the diversity of signals produced by females is particularly interesting because sexual selection on females is usually absent. Thus for allopatric populations, signal diversification via sexual selection is unlikely. Moreover, stabilizing natural selection on females’ signals in allopatry may also make diversification unlikely. However, this may not be the case if female’s sex pheromones are a mosaic of different compounds, as has been found in a number of species. In this case, while some components may be essential for male responses, and thus under stabilizing selection, selection on other components may be relaxed, and these may exhibit changes at the level of concentrations or structural changes through drift (Symonds & Elgar, 2008; Wyatt, 2003). Thus, for chemical signals produced by females phylogenetic patterns of variation may be more likely that those uncorrelated with evolutionary history.

Consistent with these ideas, the available data (for insects) have suggested two main patterns of diversification of pheromones exist across related taxa, and these are consistent
with the two processes of signal evolution that are linked to evolutionary history (Symonds & Elgar, 2008). First, pheromones may be particularly likely to undergo gradual evolution, in which small changes in pheromone composition arise from the evolutionary loss or gain of single chemical components or a change in the proportions of different components with such changes accumulating slowly over evolutionary time (Roelofs & Brown, 1982). The result of this gradual change is that closely related species have similar pheromone compositions, and the greatest divergence in chemical signals will be between the mostly distantly related species. Second, pheromones may undergo significant structural changes with the loss or gain of multiple compounds typical during diversification. In this case, more closely-related species will be more different in the chemical blends than expected by chance, suggesting selective shifts in their composition (Symonds & Elgar, 2004) most likely via selection for pre-mating isolation.

In spiders, sex pheromones play a key role in mating discrimination and assessment (Foelix, 2011). Females release cuticular and/or silk-based sex pheromones, which can be airborne or received via contact with chemoreceptors on the male’s pedipalps. Airborne pheromones primarily attract males, while contact pheromones stimulate male courtship and searching behavior (Gaskett, 2007). In many web-building spider species, when males moult to adulthood they became nomadic and start to look for females (Foelix, 2011). Males use chemical produced by females to locate conspecifics, however during searching they may also be exposed to chemicals produced by heterospecific females in the field (Foelix, 2011; Gaskett, 2007; Herberstein, 2011). In some spider species, males favour conspecific over heterospecific pheromones (Agelenidae: Roland, 1984; Trabalon et al., 1996; Salticidae: Cerveira & Jackson, 2013; Cross & Jackson 2013; Nelson et al., 2012; Willey & Jackson,
1993; Linyphiidae: Schulz & Toft, 1993), but in other species males are also attracted to pheromones produced by heterospecific females (Lycosidae: Costa & Capocasale, 1984; Robert & Uetz, 2004a,b; Stratton & Uetz 1983; Araneidae: Olive, 1982; Threchaleidae: Costa-Smith & Machado, 2012). Moreover, in one study of wolf spiders, male response to pheromone-laden silk of females was least intense for distantly related congeners, higher for closely related congeners, and highest for conspecifics (Robert & Uetz, 2004a). These patterns are consistent with the idea that female chemical signals may be conserved between closely related species, and may diverge gradually according to phylogenetic distance. However, patterns of pheromone discrimination have been studied in only a handful of spider taxa and no comparative analyses are currently available that examine patterns of signal diversity in light of a well-resolved phylogeny of evolutionary relationships, or data on genetic divergence (Gaskett, 2007; Schulz, 2013).

Here I report the results of a comparative analysis of male responses to the sex pheromones, silk, and females of six different species in the spider genus *Latrodectus*. I compared responses of males to extracted pheromones, and females and their silk within and across clades, and within and across major biogeographical regions as a function of phylogenetic relatedness, and as a function of a proxy for phylogenetic distance (genetic divergence).

The spider genus *Latrodectus* Walckenaer, 1805 (Araneae: Theridiidae) includes a group of 20 – 30 species (“widow spiders”), including the notorious ‘black widows’, with worldwide distribution, variation in ecology and degree of sympatry, but little inter-specific variation in anatomy (Garb et al., 2004) or courtship behaviour (pers. obs., Kaston, 1970),
which may suggest a history of recent, rapid speciation (see Rowe et al., 2011). Variation in the risk and fitness effects of sexual cannibalism, and costly mate searching (e.g., Andrade, 2003; Segoli et al., 2006) suggests the intensity of selection on male recognition of fertile conspecific females, mediated by pheromones, may also vary (e.g., Baruffaldi & Andrade, 2015). Molecular phylogenetic analyses identify two well-supported monophyletic clades within the genus: geometricus (mostly African) and mactans (including three sub-clades/biogeographical regions (BR: biogeographic region): South American, North America, and Australia-New Zealand) (Garb et al., 2004; Condy et al., in prep) (Fig. 2-1). Females are sedentary, while males search for and are attracted to females by airborne pheromones, and courtship commences once males touch the female’s silk (Kaston, 1970). The mating behaviour of several species of *Latrodectus* has been described, and males of some species court (and mate) heterospecific females, suggesting a conservation of contact pheromones (Kaston, 1970; Ross & Smith, 1979; Schmidt, 1991). Field work suggests a similar conservation of airborne pheromones in allopatric, distantly related species (e.g., *L. hesperus* and *L. hasselti*, Kasumovic & Andrade, 2004). There is little evidence for the type of historic geographic overlap that would be expected to lead to the divergent evolution of sexual signals in sympatry, thus here I test the hypothesis that contact sex pheromones have undergone gradual evolution, using representative species from the two clades within the genus, and from different BR within the mactans clade (Garb et al., 2004; Condy et al., in prep, Fig. 2-1). I predict sex pheromones will be more divergent in distantly related compared to closely related species, and this should be reflected in male responses to both pheromones extracted from silk and airborne pheromones, and in male courtship and mating attempts with heterospecific females.
This chapter has four goals. First, I quantify male discrimination of conspecific from heterospecific females based on contact pheromones using a silk-extract assay that measures male mate searching behavior. Second, for species of males in which there was no clear pattern of discrimination of contact pheromones, I used a T maze to test for discrimination in a 2-choice assay based on attraction to airborne (volatile) pheromones. Third, I tested whether the patterns of discrimination based on pheromones are consistent with male behaviour when all cues are present (males are placed on webs with females). And finally, I examined relationships between male sexual behaviors and the genetic divergence between the species, using data on sequence divergence in the bar coding mitochondrial gene (COI) which has proven to be very accurate for species discrimination in a range of taxa, including spiders (Barrett & Hebert, 2005).

Methods

Latrodectus biology & natural history

Similar to many other spiders, \textit{Latrodectus} females produce sex pheromones that are used by mate searching adult males to locate females and discriminate females’ reproductive status (\textit{L. hesperus}: Kasumovic & Andrade, 2004; MacLeod & Andrade, 2014; \textit{L. hasselti}: Andrade & Kasumovic, 2005). The contact pheromones of virgin females that trigger courtship by male \textit{L. hasselti} and \textit{L. hesperus} have been identified, can be extracted from the female’s silk with methanol, and include distinct but similar chemical components that trigger male sexual behaviour (Jerhot et al., 2010; Scott et al., 2015). For \textit{L. hasselti} the activity is triggered by a methyl ester of $N$-3-methylbutyryl-$O$-(S)-2-methylbutyryl-$L$-serine (Jerhot et al., 2010) and for \textit{L. hesperus} the pheromone includes a $N$-3-methylbutyryl-$O$-methylpropanoyl-$L$-serine
methyl ester, a lower homologue of the *L. hasselti* contact pheromone (Scott et al., 2015). Similarly, ongoing studies suggest the pheromone for *L. geometricus* is a N-3-methylbutyryl-O-propionyl-L-serine methyl ester (Kiefer, Baruffaldi, Andrade & Schulz, in prep). Although female silk production may vary with recent diet (e.g., Blackledge & Zevenbergen, 2007), the response of *L. hesperus* and *L. hasselti* males to silk extracts are not correlated with silk mass (Chapter 3, Baruffaldi & Andrade, 2015).

Experimental Spiders

Juveniles were reared in the laboratory from field-mated individuals of *Latrodectus hesperus* collected in California, USA in May 2011, *L. hasselti* collected in New South Wales, Australia in January 2011, *L. mirabilis* collected in Canelones, Uruguay in January 2013, *L. variolus* collected in Ontario, Canada in July 2012, *L. mactans* collected in Florida, USA in May 2012, and *L. geometricus* collected in Florida, USA December 2011. All spiders were reared individually in 5 x 5 x 7cm clear plastic containers (Amac Plastics) after the first few instars and fed twice weekly with *Drosophila sp.* fruit flies. Males were fed *Drosophila* and females (which are 100 to 200 x the body weight of males, Kaston, 1970) were fed with one cricket (*Acheta domesticus*, fed a protein-rich diet of kitten chow mixed with fish flakes) per week from approximately the fifth instar throughout adulthood. All individuals were reared under laboratory conditions, in a temperature controlled room at 25 C (12 hrs light, 12 hrs dark) and were watered once per week by lightly misting the interior of the cage.
Part 1. Contact pheromone bioassay

I measured the duration of male mate searching behavior when exposed to silk extracts from heterospecific and conspecific females as a measure of the activity and attractiveness of the sex pheromone produced by females. It is has been shown elsewhere that *Latrodectus* males show extensive activity on filter papers treated with silk extract from females and that this activity changes in intensity and duration as predicted by variation in female mating status, and diet, but not on methanol controls (Baruffaldi & Andrade, 2015; Perampaladas et al., 2008; Stoltz et al., 2007). I scored male searching behavior as total male movement in a 60 minute trial when males were placed on filter paper treated with the experimental silk extract (sex pheromone + methanol) or control (methanol alone) placed inside glass petri plates (90 mm) according to the methodology used by Stoltz et al. (2007), Perampaladas et al. (2008), Jerhot et al. (2010), and Baruffaldi & Andrade (2015). For each trial, 0.15 ml of silk extract or methanol was added to a fresh disc of filter paper using a pipette, and the filter was allowed to air-dry for 5 minutes. Males were gently placed on the filter paper and the glass lid of the arena replaced. Trials were conducted between 10am and 8pm, and recorded on digital video using Panasonic WV BP330 low-light cameras and Navitar 7000 macro-zoom lenses. As most species are nocturnal, all trials were performed during the dark phase under red lights. After each trial, the filter paper was discarded and petri plates were washed with water and ethanol, and allowed to air-dry before reuse.

To test the divergence of contact sex pheromones between clades and BR, these bioassays utilized females of 4 species representing both clades and the 3 main BR within the *mactans* clade (*L. geometricus, L. hasselti, L. hesperus*, and *L. mirabilis*, Fig. 2-1). For each
of these 4 species, naïve adult virgin males were split in 5 experimental groups and were exposed either to control (methanol) or female silk extract (sex pheromone + methanol), where the extract was from the silk of a conspecific or one of the 3 heterospecific females (4 silk-extract treatments). For these 4 species, trials were conducted using males of every species versus females of every species. Thus for each species pair, there are two sets of heterospecific response data (males of species 1 responding to females of species 2, and males of species 2 responding to females of species 1). Each male was used only once (L. geometricus n = 75, L. hasselti n = 75, L. hesperus n = 85, and L. mirabilis n = 75).

To examine divergence of sex pheromones inside the North-American BR, I did an additional set of bioassays in which I exposed L. hesperus males to extract from the silk of L. variolus and L. mactans females. Each male (n = 30) was used only once.

Silk used for extraction was produced by adult virgin females (1 - 2 months after adult moult). Females are placed in an experimental arena consisting of clean, paired, inverted U-shaped stainless-steel wires supported in a plastic block submerged in water in a larger plastic container (Baruffaldi & Andrade, 2015; Perampaladas et al., 2008; Stolz et al., 2007). In these arenas, females are restricted to the wires, across which they build horizontal webs. Each female was allowed to build a web for 4 days, after which I removed females, harvested their silk using clean glass pasteur pipettes (line glass 22.86 cm), and placed the silk in 2 ml auto-sample Teflon cup glass vials (National Scientific). Contact sex pheromone was extracted by submerging the silk in 0.15 ml of methanol (HPLC, 99.9%, Fisher Chemicals) for 24 hr (Baruffaldi & Andrade, 2015; Gaskell, 2007; Perampaladas et al., 2008;
Schulz, 2013; Stoltz et al., 2007). Between collections, all components of experimental arenas were washed with liquid soap and abundant water, and then air-dried.

Part 2. Airborne pheromones

Females and their silk were used as sources of airborne pheromones for males tested in a two-choice T-maze arena. Conducted on one representative species from the mactans clade (*L. hasselti*), this was intended to allow comparison of responses to airborne and contact pheromones. Adult virgin females from *L. hasselti*, *L. mirabilis*, *L. geometricus* and *L. hesperus* were allowed to build webs for 7 days in rectangular experimental cages (8 x 8 x 12 cm) with walls constructed of dark screen to allow dissipation of the airborne sex pheromones, but which prevented direct contact with males. Experimental arenas were large plastic rectangular boxes (1m x 0.5m) with T-mazes placed inside, parallel to the long-axis of the arena. T-mazes were constructed of 1 cm diameter bamboo sticks, and were wiped with water after each trial. The end walls were replaced by dark screen with small fans (output: +5.0 V, 2.5 A) blowing gently into the arena. Two experimental cages with females (and their silk) from different species were placed just inside the end walls, one at each terminal end of the T-maze. Males were carefully placed at the top of the cross-arm and behavior was analyzed for 10 minutes. I recorded the male’s location continuously (time spent on either arm of the maze, or on the stimulus cages) and also made note of which of the stimulus cages the male contacted first (‘choice’) and how much time the male spent on each stimulus cage. In this repeated measures design, each *L. hasselti* adult virgin male was tested in both a control/conspecific and a conspecific/heterospecific scenario (4 treatments in total, with 3 possible heterospecific stimuli, one of which was assigned randomly to each male). The two
treatments were presented in random order to each male, with a 24 h interval between treatments. Experimental (conspecific or heterospecific females) and control (empty) cages were alternated in their position in order to avoid a male bias towards any particular direction. All the experiments were conducted in an environment-controlled room that contained no other spiders or cages. Trials were conducted during the spider’s dark phase under red light. All the cages and maze components were washed and the silk was removed between trials.

Part 3. Laboratory matings

Focal adult virgin males were paired individually with conspecific or heterospecific adult virgin females mirroring the representative species used in the contact sex pheromone experiment (Part 1, thus data include reciprocal male-female matings for all species pairs). All pairings occurred on female’s webs and were recorded with digital video for an 8 hour period or until males were killed by females (using well-worked out methodology: Stolz et al., 2008, Chapter 4). I analyzed the frequency of courtship and mating success (male palpal insertion onto female genitalia) and fitness effects (offspring production) of hybridization.

Part 4. Genetic Distance and behaviour.

The gradual evolution hypothesis predicts that genetic divergence between focal species should predict male responses to heterospecific females and their pheromones (i.e., male searching activity on female silk extracts, male courting behaviour and heterospecific mating success). I tested this prediction using data on genetic divergence (% difference) in COI sequences of focal species pairs. Genomic DNA extractions were completed using muscle
tissue (from up to four legs) using DNeasy Extraction Kit (Qiagen Inc). PCR was used to obtain 505bp of cytochrome c oxidase subunit 1 (COI) fragments. PCR products were purified and then sent to Sick Kid’s Hospital in Toronto for sequencing. Sequence alignments were generated using Clustal W within Geneious 6.17 (Kearse et al., 2012) (with a gap opening and extension cost combination of 10/10). The resulting alignment was used to quantify gene sequence divergence in pairs of included species, and this value was used as my measure of phylogenetic divergence.

Statistical analysis

Analyses were completed in IBM SPSS Version 22 (SPSS Inc., Chicago, IL, U.S.A.). Male behavioural data were not normally distributed in some of our behavioural and activity categories (Shapiro-Wilk tests of normality), and data transformations were unsuccessful. I thus used Generalized Linear Models (GLMS) with Gamma log link distribution for analyzing durations (treatment = fixed factor; behaviours = dependent variables), and the relationship between behavior and genetic distance. Post hoc least-significant difference tests were also performed to examine how male response to conspecifics compared to heterospecific females and controls. For the airborne pheromone experiments, χ² tests were used to analyze the frequency of male preferences.

Results

Part 1. Contact pheromones

In all cases, males spent more time searching on solvent extracts than controls (Fig. 2-2). L. geometricus males (n = 75) showed the highest species-specificity responses, spending
significantly more time searching on silk extracts from conspecific females than on extracts from any of the heterospecific females (Fig. 2-2A). *L. hesperus* males (n = 75) were intermediate, as they responded to *L. hasselti* extracts, although at lower level than to conspecifics, but not significantly so. Moreover, although these males responded less to *L. mirabilis* females than to conspecifics, this difference was only borderline significant (Fig. 2-2D; p = 0.049), however they did not respond to silk extract from *L. geometricus* females (Fig. 2-2D). In contrast, *L. mirabilis* (n = 75) and *L. hasselti* (n = 75) males clearly showed the lowest degree of species-specificity. *L. mirabilis* males spent very similar search times on silk extract from conspecifics and *L. hasselti* females (Fig. 2-2B), and *L. hasselti* males had similar search times on silk extract from conspecific, *L. mirabilis* and *L. geometricus* females (Fig. 2-2C).

Within the mactans clade, *L. hesperus* males were also tested on silk extracts from two other North-American species. Males showed similar responses to extracts from conspecifics, *L. various* and *L. mactans* (Fig. 2-3).


Similar to the contact pheromone results, *L. hasselti* males were equally likely to be attracted to airborne chemicals from *L. mirabilis* or *L. geometricus* females relative to conspecific females, and spent equivalent time searching outside cages of their choice, regardless of species (Fig. 2-4A, B). In contrast, when the choice was between a conspecific and *L. hesperus*, males chose conspecific females 76% of the time. However, males that chose to move to an *L. hesperus* female’s cage spent equivalent time searching there as did males
outside of conspecific females’ cages (Fig. 2-4C). Finally, as expected, *L. hasselti* males chose conspecifics over empty controls ($\chi^2 = 8.77, p = 0.003$).


Most males courted, and tried to mate with heterospecific females. Males from the two species that showed lower discrimination of pheromones across clades (i.e., *L. hasselti, L. mirabilis*) had higher mating success on average than did the males that showed higher discrimination (*L. hesperus, L. geometricus*; Table 2-1). No heterospecific pairings produced viable progeny.

Part 4. Genetic Distance and behavior.

As predicted, male response to extracted pheromones increased as genetic distance between species pairs decreased (GLM: likelihood ratio: $\chi^2 = 20.844, p = 0.004$, Fig. 2-5, Table 2-2). However male courtship on female’s webs showed the opposite relationship, with males courting less as genetic distance between species pairs decreased (GLM: likelihood ratio: $\chi^2 = 22.410, p = 0.002$, Fig. 2-5, Table 2-2). There was no significant relationship between male mating success and genetic distance (GLM: likelihood ratio: $\chi^2 = 13.221, p = 0.067$, Fig 2-5, Table 2-2) although mating success with heterospecific females ranged from 0 to 76% of conspecific mating rates.

These comparisons are complicated by two aspects of the data (outlined below), and so I also report alternative treatments that sub-sample the data to reduce effects of these issues (Table 2-2).
First, some species pairs share a more recent phylogenetic history than others, and so every comparison may not be statistically independent. This is particularly true for the species within the North American SC/BR (L. hesperus, L. mactans and L. variolus), which are also the species with the lowest genetic distances. I re-examined the data while sequentially excluding 2 of the 3 comparisons for this SC/BR (Table 2-2). I found the same significant (negative) relationship between male response to extracted pheromones and genetic distance regardless of whether I used all the data or a subset (Table 2-2). However, when L. mactans was excluded from the analysis, the (positive) relationship between courtship and genetic distance was lost (Table 2-2).

Second, most inter-species comparisons include reciprocal assessments of response (e.g., males of species 1 vs. females of species 2 and vice versa) and thus, for most of the data, each species pair is represented by 2 data points (which are sometimes quite different, see HA-MI vs. MI-HA, Fig. 2-5). Within the North American SC/BR however, only one point is available for each species pair (male L. hesperus tested against females of the other 2 species). Thus I re-analyzed these data including only the species for which I have paired data. I found again that male response to extracted pheromones increased as genetic distance between species pairs decreased (GLM: likelihood ratio: $\chi^2_5 = 13.883$, $p = 0.016$, Fig. 2-5, Table 2-2), however I did not find any correlation with courtship behavior nor male mating success (Table 2-2).

**Discussion**

There are two clades within *Latrodectus*, with one (geometricus) represented by only a few species found in Africa, and the second (mactans) including all other species, with sub-clades
within *mactans* suggesting more recently derived biogeographic clusters (SC/BR) of diversity. I used representative species to test predictions of a pattern of gradual evolutionary change; I expected that if phylogenetic relatedness and genetic distance underlies diversity in sex pheromone attraction and/or composition between species, *L. geometricus* males would show the most discrimination of heterospecifics, but more recently diverged species, such as those in the North American SC, would be much less so (Fig. 2-1). Supporting this prediction, *L. geometricus*, the species representing the deepest division in the genus, was the only species in which males searched on extracts of silk from conspecific females only (Fig. 2-2). In contrast to *L. geometricus*, *L. hesperus* males showed less discrimination. Although active on extracts from conspecific females, they were equally active in searching on silk extract from other North-American species (same SC/BR), and also showed responses to silk extract from *L. hasselti* and *L. mirabilis* (same clade, different SC/BR). However, as predicted, male *L. hesperus* did not respond to silk extract from *L. geometricus* females (different clade). Moreover, there is a general pattern of increased male response to pheromones as genetic distance between species decreases (Fig. 2-5). Taken together, these results provide support for the prediction that more closely related species should present more similarities in their chemical attractiveness or pheromone composition.

One particularly interesting result of this study is that, *L. mirabilis* males responded strongly to silk extract from *L. hasselti* females, and *L. hasselti* males showed strong responses to silk extract from *L. mirabilis*. These responses are not inconsistent with genetic distance predictions, however, since *L. hasselti* and *L. mirabilis* show relatively high sequence similarity (Fig. 2-5). In fact, outside of the North American SC/BR, this species pair showed the highest similarity of any comparison in our sample (Fig. 2-5). Moreover,
very similar patterns of attractiveness were obtained in the tests of the response of *L. hasselti* males to airborne pheromones (Fig. 2-4). This increases confidence in the contact pheromone results and also suggests that contact and airborne pheromones could transmit the same information (Baruffaldi & Andrade, 2015; MacLeod & Andrade, 2014). This leads to the intriguing question of whether the airborne pheromone is in fact a volatile component of the contact pheromone (e.g., Papke et al., 2001) or whether both are different signals but with redundant information (Zuk et al., 1992). Taken together, these data suggest that the gradual divergence hypothesis seems to apply across the genus.

These results have interesting implications for understanding patterns of current distribution and invasiveness. First, in this study, the species most efficient at discriminating heterospecific females was not only from a lineage arising from the deepest division in the phylogeny, but is also the most invasive *Latrodectus* with a worldwide distribution, *L. geometricus*. Selective pressures on *L. geometricus* could be very high for mate discrimination given their modern distribution, which includes extensive areas of sympatry with a number of other species. It is interesting to note that *L. hasselti*, the other species known to be invasive (Forster, 1984; Vink et al., 2008, 2011), shows less discrimination. However, to date it has extended its range only where no other *Latrodectus* species exist (across Australia, Japan), or where a single congener exists (New Zealand). For *L. hasselti* in New Zealand, inter-breeding appears to be common, but directional (male *L. hasselti* mate with female *L. katipo*, but not vice-versa), leading to directional introgression of hasselti genes into katipo populations, and possibly contributing to the ‘at risk’ status of *L. katipo* in regions of overlap (Vink et al., 2008). Although earlier studies suggest this directional effect may be partly mediated by differential female responses to heterospecific males (*L. hasselti*
females are discriminating, *L. katipo* females are not), my study suggests this may also be mediated by pheromones. For most species pairs examined here, reciprocal tests showed differences in male responses—so for example, males of species 1 showed higher responses to females of species 2 than did males of species 2 to females of species 1. Given that differences in pheromone structure must be the same for both of these, this result shows that male discrimination thresholds vary among species.

Despite the consistent phylogenetic signal in male responses to pheromones, when males were placed on females’ webs, most of the males (75% of all the males used) courted heterospecific females, and some of the specificity in responses to the pheromones alone seems to be absent (Table 2-1, Fig. 2-2). However, males could be doing courtship displays not only for mating with a female, but also as a way to advertise that they are males and not prey entangled on the web (Foelix, 2011; Huber, 2005). Though males may be exhibiting courtship to avoid being killed by females, 20% of all males tested tried to copulate with heterospecific females, showing recognition of a potential mate. For example, with *L. geometricus* males, mating was most frequently successful with *L. hasselti*. However this may suggest similarity of mating sequence since they are the only two species used here with a unique copulatory behaviour (male ‘somersault’ behavior, Andrade, 1996, 1998; Segoli et al., 2008). In comparison, consistent with predictions based on phylogenetic distance, male *L. geometricus* exhibited very low mating success with *L. hesperus* and no successful matings with *L. mirabilis* females (Table 2-1, Fig. 2-1). Contrary to this hypothesis, and surprising given the pheromone results, *L. hesperus* males showed low mating success with other North American species (consistent with previous observations (Kaston, 1970), but relatively high success only with *L. hasselti* (same clade, but different SC/BR, Table 2-1). *L. mirabilis* and
*L. hasselti* mated with all other the species (Table 2-1). However, none of the inter-species matings (N=304) produced viable offspring, suggesting a post mating block to hybridization.

Intriguingly, the results of mating trials show clearly that other information (similarities in web structure, physical properties of the silk, female anatomy or behavior, Foelix, 2011; Kaston, 1970) are also important in triggering mating behavior in males (Table 2-1), and that courtship components may be broadly conserved. This suggests that the risk of forgoing a virgin female which does not smell or taste quite right, but is sufficiently recognizable from other cues (silk, behaviour) when in close proximity, could be strong enough to ignore the information provided by the chemical signals alone. Males searching for females are exposed to high predation risk, with more than 80% of males dying during mate searching in *L. hasselti* (Andrade, 2003). Hence, in *L. hasselti*, most males will die without finding a female, and for that reason they may experience high selective pressures to attempt to mate with any female that they are able to find. It is possible that there are similar dynamics other species used in this study (e.g., similarly high mortality has been measured in two other *Latrodectus* species: Segev et al., 2003; Segoli et al., 2006). Moreover, since males courted in almost of all of these pairings, it is likely some of the differences in mating success are driven by variation in females’ behaviour or in inter-sexual interactions. These analyses are beyond the scope of this paper, but are addressed in my ongoing research.

Conclusions

My results show that widow males in general present a phylogenetic pattern of discrimination when exposed to sex pheromones produced by heterospecific females. Most of the males were attracted to heterospecific pheromones produced by closely related species.
However, more species pairs need to be added in future work, particularly from sympatric populations, or species with less genetic distance. More information about the chemical structure of the pheromones are also necessary to infer patterns of signal evolution. Nevertheless, this study is one of the most comprehensive analysis of this type available for invertebrates, and an important addition to our broader cross-taxon understanding of signal divergence, and pre and post mating divergence (Coyne & Orr, 1997).
References


**Figure 2-1.** Phylogeny of 23 of the 31 Latrodectus species recovered from a Bayesian analysis of a 505bp of CO1 gene sequence analysis modified from (Condy et al., in preparation). Focal species for these studies are highlighted with boxes and include one representative from the geometricus clade (brown), and 5 from the mactans clade, distributed across three representative sub-clades that correspond to distinct biogeographical regions SC/BR (South American, purple; North American, black, Australia-New Zealand, red). Bayesian posterior probabilities are shown above each node with the well-supported nodes for the two clades and focal SC/BR within the mactans clade highlighted (*). The topology of this phylogeny with respect to the focal species is qualitatively similar to that published previously based on a smaller taxonomic sample (Garb et al., 2004), and to that derived from a preliminary multi-gene analysis (Condy et al., in prep).

**Figure 2-2.** Duration (mean ± standard error) of searching behavior of male *Latrodectus* spiders in response to silk extract from the web of females from 4 species used to test divergence among clades and sub-clades/biogeographical regions the mactans clade. Naïve virgin males of *L. geometricus* (A), *L. mirabilis* (B), *L. hasselti* (C) and *L. hesperus* (D) were tested on silk extracts from virgin female representatives of all 4 species and a solvent-only control, with 15 (A-C) or 17 (D) males tested on each type of extract. Generalized linear models with gamma distribution and log link were used to determine whether the species of the female affected male responses (GLM: (A) likelihood ratio: $\chi^2_{4} = 16.880, p = 0.002$, (B) likelihood ratio: $\chi^2_{4} = 18.904, p = 0.001$, (C) likelihood ratio: $\chi^2_{4} = 23.636, p < 0.001$, (D) likelihood ratio: $\chi^2_{4} = 9.993, p = 0.041$). Pairwise least significant difference test between male response to conspecific females and the other treatment groups are denoted by brackets and p-values above columns.

**Figure 2-3.** Duration (mean ± standard error) of *L. hesperus* male searching behavior in response to silk extract from the web of females from two other North American species. Pairwise least significant difference tests comparing male response to conspecific females and the other treatment groups are denoted by brackets and p-values above columns.

**Figure 2-4.** The time spent on conspecific and heterospecific females’ cages for male *L. hasselti* placed in a two-choice T-maze apparatus that tested for variation in male response to airborne pheromones from females. In each case males were tested against airborne chemicals from a conspecific and a heterospecific (i.e., *L. mirabilis*, [A] N=25; *L. geometricus* [B] N=25; *L. hesperus* [C] N=21). Males were either attracted to the conspecific first (black circles) or to the heterospecific female first (white circles), but once at a cage, males typically spent the rest of the trial there. $\chi^2$ tests
were used to analyze the frequency with which males chose one type of cage first, and a generalized linear model with gamma log distribution was used to analyze the time spent on each female’s cage. In [A] 12 *L. hasselti* males chose the experimental cage with *L. mirabilis* females and 13 chose conspecific females ($\chi^2 = 0.040, \ p = 0.84$), moreover males spent similar time searching on the cage of chosen females, regardless of the species chosen (GLM: likelihood ratio: $\chi^2_1 = 0.002, \ p = 0.965$). In [B] 11 *L. hasselti* males chose the experimental cage with *L. geometricus* females and 13 chose conspecific females ($\chi^2 = 0.167, \ p = 0.683$), 1 did not move, and males spent similar time on cages of conspecific and heterospecific females (GLM: likelihood ratio: $\chi^2_1 = 0.430, \ p = 0.512$). In [C] 5 *L. hasselti* males chose the experimental cage with *L. hesperus* females and 16 chose conspecific females ($\chi^2 = 5.762, \ p = 0.016$), however they spent similar time on the cages of the females that were chosen (GLM: likelihood ratio: $\chi^2_1 = 0.039, \ p = 0.843$). Finally, as expected, *L. hasselti* males chose conspecifics over controls (not shown: $x^2 = 8.77, \ p = 0.003$).

**Figure 2-5.** Genetic distance (% sequence difference in a 505 bp fragment of cytochrome c oxidase subunit 1) between pairs of sampled *Latrodectus* species compared to male behavioural responses to heterospecific compared to conspecific silk extract [A] or females [B, C]. Male responses to heterospecifics are expressed as a proportion of the measured response to conspecifics (i.e., if responses to heterospecifics and conspecifics are equal, score is 1.0). Each point represents the average outcome of pairings between species used to test divergence among clades and sub-clades (see Figures 2, 3, Table 1), in which the first notation represents the male and the second the female (HA: *L. hasselti*, HE: *L. hesperus*, MI: *L. mirabilis*, GE: *L. geometricus*, V: *L. various*, MA: *L. mactans*).
Figure 2-1

A phylogenetic tree illustrating the evolutionary relationships among different species of Lepidoptera. The tree is divided into two main clades: the *geometricus* clade and the *mactans* clade. The tree highlights the species distribution in North America, South America, and New Zealand, with specific emphasis on the *hasselti* species in New Zealand.
Figure 2-2

(A) L. geometricus male mean searching behaviour (min)

(B) L. mirabilis male mean searching behaviour (min)

(C) L. hasselti male mean searching behaviour (min)

(D) L. hesperus male mean searching behaviour (min)

Control L. hesperus L. mirabilis L. hasselti L. geometricus

Control L. hesperus L. mirabilis L. hasselti L. geometricus
Figure 2-3
Figure 2-4
Table 2-1

Frequency of courtship and mating of pairs of males and females in 6 species of *Latrodectus* spiders, representing two clades and the major biogeographical regions/sub-clades within the genus (geometricus clade: *L. geometricus*; mactans clade: *L. hasselti* [Australasia], *L. mirabilis* [South America], *L. hesperus, L. variolus, L. mactans* [North America]) with the success of conspecific pairings (frequency of male courtship/frequency of mating) in bold on the diagonal and courtship and mating frequency of heterospecifics normalized by conspecific scores.

<table>
<thead>
<tr>
<th></th>
<th><em>L. geometricus</em></th>
<th><em>L. hasselti</em></th>
<th><em>L. mirabilis</em></th>
<th><em>L. hesperus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. geometricus</em></td>
<td><strong>100%/50%</strong></td>
<td>100/12%</td>
<td>35/5%</td>
<td>72/0%</td>
</tr>
<tr>
<td><em>L. hasselti</em></td>
<td>100/60%</td>
<td><strong>90%/80%</strong></td>
<td>80/72%</td>
<td>83/8%</td>
</tr>
<tr>
<td><em>L. mirabilis</em></td>
<td>78/0%</td>
<td>94/25%</td>
<td><strong>100%/80%</strong></td>
<td>81/0%</td>
</tr>
<tr>
<td><em>L. hesperus</em></td>
<td>85/10%</td>
<td>100/50%</td>
<td>42/23%</td>
<td><strong>90%/75%</strong></td>
</tr>
<tr>
<td><em>L. variolus</em></td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>44/0%</td>
</tr>
<tr>
<td><em>L. mactans</em></td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>17/13%</td>
</tr>
</tbody>
</table>
Table 2-2

Result of generalized linear models assessing the relationship between responses of *Latrodectus* males to silk extracts or females and genetic distances between species pairs (% sequence difference in a 505 bp fragment of cytochrome c oxidase subunit 1). The first analysis (1) includes all data for the six focal species used in this paper. The following two models (2,3) sequentially excluded 1 of the species within the North American SC/BR (*L. mactans* and *L. variolus*) because they share a recent phylogenetic history, and the final model (4) includes only data for species pairs in which males of each species were tested against females of the other species and vice versa (reciprocal data). Bolded results are statistically significant.

<table>
<thead>
<tr>
<th></th>
<th>Male mate searching behaviour vs genetic distance</th>
<th>Courtship on female’s web vs genetic distance</th>
<th>Male mating success vs genetic distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. All species included</td>
<td>GLM: likelihood ratio: $\chi^2_7 = 20.844, p = 0.004$</td>
<td>GLM: likelihood ratio: $\chi^2_7 = 22.410, p = 0.002$</td>
<td>GLM: likelihood ratio: $\chi^2_7 = 13.221, p = 0.067$</td>
</tr>
<tr>
<td>2. Without <em>L. mactans</em></td>
<td>GLM: likelihood ratio: $\chi^2_6 = 17.696, p = 0.007$</td>
<td>GLM: likelihood ratio: $\chi^2_6 = 11.283, p = 0.080$</td>
<td>GLM: likelihood ratio: $\chi^2_6 = 12.273, p = 0.056$</td>
</tr>
<tr>
<td>3. Without <em>L. variolus</em></td>
<td>GLM: likelihood ratio: $\chi^2_6 = 17.696, p = 0.007$</td>
<td>GLM: likelihood ratio: $\chi^2_6 = 19.709, p = 0.003$</td>
<td>GLM: likelihood ratio: $\chi^2_6 = 9.330, p = 0.156$</td>
</tr>
<tr>
<td>4. Only species pairs with reciprocal data sets</td>
<td>GLM: likelihood ratio: $\chi^2_5 = 13.883, p = 0.016$</td>
<td>GLM: likelihood ratio: $\chi^2_5 = 7.676, p = 0.175$</td>
<td>GLM: likelihood ratio: $\chi^2_5 = 8.535, p = 0.129$</td>
</tr>
</tbody>
</table>
Chapter 3

Contact pheromones mediate male preference in black widow spiders: avoidance of hungry sexual cannibals?

Abstract

Males often exercise mate choice when mating frequency is constrained, costs of choice are low and variation in female quality and/or expected paternity can be reliably detected. Across invertebrates, males use sex pheromones to discern female mating status, but there are few demonstrations that information about expected fecundity (‘quality’) is encoded in pheromones alone. Here I examine whether females’ sex pheromones allow males to detect differences in female food intake and mass in two species of widow spiders (Latrodectus hesperus and Latrodectus hasselti) in which chemicals are deposited by females in silk. Recent work shows that male L. hesperus prefer well-fed females, and that these females produce more silk than hungry females. Thus, changes in diet could be mechanistically linked to changes in silk-bound pheromonal signals. I show that unmated females of both species lose more than half of their mass when food is withheld, and silk production is reduced by 48% (L. hesperus) to 67% (L. hasselti). Males had a significant sexual response to pheromones extracted from the females’ silk in both species, although this response was not directly correlated with silk or female mass. In L. hesperus, but not in L. hasselti, males were less responsive to sex pheromones from food-deprived females compared to well-fed females. While females on good diets provide the benefit of higher fecundity in both species,
the risk of being cannibalized by hungry females during courtship exists only in *L. hesperus*. I conclude that sex pheromones alone can provide information about recent female feeding history, possibly reducing the costs of males expressing choice in the field. The species difference in male response also suggests that male preferences in these spiders may depend less on the benefit of seeking a highly fecund female and more on avoiding the cost of risky mating attempts with a likely cannibal.
Introduction

A variety of studies now show that male mate choice often co-occurs with intermale competition in species where females invest more in offspring than do males (reviewed in: Bonduriansky, 2001; Edward & Chapman, 2011). As with female choice, the evolution and maintenance of male mate choice is more likely when variation in mate quality is high, the cost of being choosy is low, and when investment in each reproductive opportunity, whether this is via mating effort (investment in securing copulations/fertilizations) or paternal effort (investment in offspring), is sufficient to limit mating frequency (Bonduriansky, 2001; Edward & Chapman, 2011; Jennions & Petrie, 2000). However, mate choice may differ between the sexes in ways that could have important implications for the evolution of choosy males. Mate choice critically depends on the ability to discriminate the relative value of a potential partner, and there may be sex differences in the availability of reliable cues of mate quality. For choosy females, discrimination is often facilitated by ornaments or displays that correlate with male traits and evolve through sexual selection on competing males (Candolin, 2003). In contrast, sexual selection on females is typically weak (Andersson, 1994), so choosy males will often need to discriminate among females for which natural selection tends to favour crypsis over display (Arnqvist & Rowe, 2005). If cues related to female quality are readily discriminable, the cost of being choosy will decrease and the potential fitness benefits required for choice to be a viable tactic will also decrease. Thus, the conditions under which we expect to find male mate choice in nature can be quite variable, and understanding male mate choice requires not only identifying the particular qualities
preferred by males (male preferences, Jennions & Petrie, 2000), but also which cues males may use to accurately identify preferred females.

Although confidence of paternity is clearly critical for choosy males (reviewed in Edward & Chapman, 2011), male fitness is also limited by the reproductive potential (‘quality’) of his mates. Considerable theoretical and empirical work confirms that, in terms of male preferences, the most relevant aspect of female quality across taxa is fecundity (or fertility, Bonduriaski, 2001; Edward & Chapman, 2011). At a given risk of sperm competition, the benefits of having a highly fecund mate may be sufficient to favour choice, particularly if being choosy does not in itself cause significant additional restrictions on mating frequency in nature (Edward & Chapman, 2011). Ovum production (maximum fecundity) is often positively correlated with female body mass or size (e.g. amphibians: Verrell, 1985; fish: Kraak & Bakker, 1998; invertebrates: Danielson-Francois et al., 2002; Harari et al., 2011; Johnson et al., 2014; Lelito & Brown, 2008). Moreover, body mass or size may reflect recent food intake, which can affect the likelihood and timing of reproduction (realized fecundity), with poorly fed females delaying egg laying (Stoltz et al., 2010). While mass, size, and even mating status could be assessed visually in some taxa (e.g. Maxwell et al., 2010a; Maxwell et al., 2010b), in many others this is not possible and this information would have to be gleaned from alternative modalities (e.g. Allen et al., 2012). For example, chemical signals or cues can allow males to assess female fecundity and mating status (e.g. Barry, 2010; Chinta et al., 2010; Foster & Johnson, 2011; Harari et al., 2011; Lelito & Brown, 2008; Maxwell et al., 2010b; Prouvost et al., 1999) or receptivity (Maxwell et al., 2010a), and this assessment may be accomplished at a distance, allowing males to assess females prior to heavy investment in courtship (Barry, 2010; Harari et al., 2011; Johansson &
Jones, 2007; Maxwell et al., 2010a; Maxwell et al., 2010b; Thomas, 2011), thus significantly reducing the cost of choice. Chemical communication is common in invertebrates, so male mate choice could be widespread if female fecundity and mating status are commonly encoded in sex pheromones, and if male preferences (Jennions & Petrie, 2000) are based on this information (e.g. Gaskett, 2007; Johansson & Jones, 2007; Thomas, 2011). If so, given their diversity, invertebrates may be particularly interesting models for assessing male mate preferences, and for testing general theory regarding the evolution and maintenance of male mate choice.

Here I focus on male assessment of female feeding history, examining whether sex pheromones alone permit male discrimination of mates in two species of ‘widow’ spiders (*Latrodectus hesperus* and *Latrodectus hasselti*, Araneae: Theridiidae), and consider whether patterns of discrimination can suggest the underlying traits that determine male preferences in these species. In spiders, male choice may be beneficial because mating frequency can be limited by male exposure to high predation risk during mate searching (e.g. *Latrodectus*: Andrade, 2003; Segoli et al., 2006; *Nephila*: Kasumovic et al., 2007) and courtship (e.g. *Argiope*: Herberstein et al., 2002), in addition to which mating often includes the risk of sexual cannibalism (Huber, 2005). Mate discrimination may be possible in spiders because females produce sex pheromones on their bodies or silk, and these can be used by males to localize potential mates, to trigger courtship and to assess female phenotypes and mating status (Baruffaldi & Costa, 2010, 2014; Gaskett, 2007; Uhl & Elias, 2011). Males of many spider species show variation in courtship intensity in response to silk threads and/or silk extracts of females that vary in mating status (Baruffaldi & Costa, 2010, 2014; Gaskett et al., 2004; Roberts & Uetz, 2005; Rypstra et al., 2003; Stoltz et al., 2007). However, most tests
for cues of female fecundity and recent feeding history have used the female, her silk, or both to test male responses (e.g. Johnson et al., 2011; MacLeod & Andrade, 2014; Schulte et al., 2010). Here I focus on establishing a causal link between male behaviour and pheromonal cues of fecundity or size by manipulating female diet and assaying male responses to silk extracts in the absence of other types of information (see Gaskett, 2007; Schulz, 2013). Responses to sex pheromones are a commonly used proxy for mate preference since such responses mediate mate attraction, courtship and coupling in nature in many invertebrates (Johansson & Jones, 2007; Thomas, 2011).

In recent studies conducted in western black widow spiders (L. hesperus) males were found to adjust their mating effort based on web-borne cues of female feeding history (in the laboratory, Johnson et al., 2011), and were preferentially attracted to females and their webs if those females are well fed and unmated (in the field, MacLeod & Andrade, 2014). This shows that males are choosy in nature (MacLeod & Andrade, 2014); that is, they act on preferences in the presence of natural costs (e.g. Jennions & Petrie, 2000). This also suggests that male preferences for heavier females could be mediated by chemical cues. However, in both studies, other cues in addition to chemicals from the silk were present (bodies of prey, and females), leaving it unclear whether chemical cues were responsible for male choice. Here I asked how female mass and silk production is affected by diet, and whether males discriminate between well-fed and poorly fed females based on silk-borne chemical cues (sex pheromones) alone. There are mechanistic links between diet and silk production in L. hesperus that may facilitate such discrimination; well-fed females produce more silk and alter the structure of the web relative to starved females (Blackledge & Zevenbergen, 2007). Thus, hungry females may produce less pheromone-laden silk than well-fed females. I also
assess whether there is a link between diet and silk volume in *L. hasselti*, a congener in which this has not previously been examined. This is a first step toward asking whether this is a genus-wide response by females to variable food intake.

Male spiders may also be choosy because of the risk of being attacked and consumed by females, and this is particularly true when cannibalistic females typically attack males that are courting (‘precopulatory sexual cannibalism’, Elgar, 1992, 1998; Elgar & Schneider, 2004; Herberstein et al., 2002; Huber, 2005). In this case, selection to detect and avoid females with a high propensity to cannibalize should be strong (e.g. Johnson et al., 2011; Maxwell et al., 2010a). This is consistent with patterns of male preference for satiated females reported for praying mantids where the risk of precopulatory cannibalism is high, particularly when females are poorly fed (Lelito & Brown, 2006, 2008; Liske & Davis, 1987). This prediction, however, is not as strong if cannibalism occurs during or after mating and cannibalized males transfer sperm successfully. In this case cannibalized males can still accrue paternity, and in some species, actually have higher paternity than males that are not cannibalized (e.g. *Latrodectus*: Andrade, 1996; *Argiope*: Schneider et al., 2006).

I was particularly interested in whether male preference for well-fed females (Johnson et al., 2011; MacLeod & Andrade, 2014) is more likely explained by the fecundity benefits of mating with these females, or by the costly risk of being cannibalized by poorly fed females. These hypotheses are not exclusive, and disentangling these possible fitness benefits is challenging. I approached this puzzle by considering how cannibalism and fecundity benefits might affect the maintenance of male preferences in *L. hesperus* and *L. hasselti*. Although sexual cannibalism occurs during courtship and is costly to males in *L.*
*hesperus* (Johnson et al., 2011), it is coincident with sperm transfer and part of the male’s sexual strategy in *L. hasselti* (Andrade, 1996; Forster, 1992). If male preference for well-fed females is maintained primarily because of the decreased risk of sexual cannibalism, I predicted that male *L. hesperus*, but not *L. hasselti*, would show a preference for well-fed females. On the other hand, if choosy males have higher fitness because this allows them to mate with more fecund females and fertilize more eggs, I would expect to find this preference in both species. Although this approach does not allow us to examine explanations for the origins of male preferences, it can suggest which hypothesis is most consistent with the microevolutionary processes that would be necessary to yield variation across species in mate discrimination.

**Methods**

Latrodectus Biology and Natural History

Like many other web-building spiders, *L. hesperus* and *L. hasselti* females are sedentary and build or repair their webs each night. Female size, mass and/or recent diet predicts reproductive output in *L. hesperus* (MacLeod, 2013) and *L. hasselti* (Stoltz et al., 2010). In contrast to females, nomadic adult males leave their own web and use females’ chemical signals to search for mates and to discriminate among females when searching (*L. hesperus*: Kasumovic & Andrade, 2004; MacLeod & Andrade, 2014; *L. hasselti*: Andrade & Kasumovic, 2005) and when in contact with silk or silk extracts (*L. hasselti*: Andrade & Kasumovic, 2005; Stoltz et al., 2007). The pheromone of virgin females that triggers courtship by male *L. hasselti* when they contact the web is an acylated serine derivative that
Experimental Spiders

Juveniles were reared from laboratory populations of *L. hesperus* (Chamberlin & Ivie, 1875) whose parents were field-mated individuals collected near Monterey, California, U.S.A. in May 2009, and *L. hasselti* (Thorell, 1870) collected in Sydney, New South Wales, Australia in January 2010. All spiders were reared individually in 5 × 5 × 7 cm clear plastic containers (Amac Plastics) after the first few instars and fed twice weekly with *Drosophila* sp. fruit flies. Males were fed *Drosophila* throughout development and females (which are 100–200× the body weight of males, Kaston, 1970) were fed one cricket (*Acheta domesticus*, maintained on a protein-rich diet of kitten chow mixed with fish flakes) per week from approximately the fifth instar until they became adults. On this diet, spiders mature and mate normally in the laboratory, and for *L. hasselti*, adult mass is comparable to that of field-collected females (Stoltz et al., 2010) and males (Andrade, 2003; Kasumovic & Andrade, 2006). All the individual were reared under laboratory conditions, in a temperature-controlled room at 25 °C (12:12 h light:dark cycle).

Experimental Females

I compared male responses to sex pheromones extracted from silk as a function of the female’s diet. One day after their final moult, experimental females were split randomly in two diet treatment groups: fed (*N* = 25 *L. hasselti*; *N* = 25 *L. hesperus*) and unfed (*N* = 25 *L. hasselti*; *N* = 22 *L. hesperus*). Fed females were given one cricket per week (normal diet in
the laboratory), while unfed females were left without food for 4 weeks. Previous studies showed that 4 weeks of diet treatment is sufficient to cause male discrimination of females and their webs (Johnson et al., 2011), most female Latrodectus can recover from this duration of food deprivation (Forster & Kavale, 1989), and similar periods of food deprivation may not be uncommon in nature (Stoltz et al., 2010). All females used in these experiments recovered after 4 weeks of food deprivation and were returned to our laboratory population and placed on normal diets at the conclusion of the experiment.

Silk Collection and Pheromone Extraction

After the 4-week period, females from both treatments were weighed (Ohaus electronic balance, accurate to 0.01 mg), and each was allowed to build a web for 4 days in an experimental arena consisting of clean, paired, inverted U-shaped stainless-steel wires supported in a plastic block submerged in water in a larger plastic container. After this period, I removed females and harvested the silk produced using glass Pasteur pipettes (line glass 9’’ and placed the silk in 2 ml auto-sample Teflon cup glass vials (National Scientific, Rockwood, TN, U.S.A.). Sex pheromone was extracted by submerging the silk in 0.2 ml of methanol (HPLC, 99.9%, Fisher Chemicals) for 24 h, following established protocols (Gasket, 2007; Stoltz et al., 2007). After extraction, the silk was removed from the vial and allowed to dry completely, then weighed (Ohaus electronic balance, accurate to 0.00001 mg). Between collections, all components of experimental arenas were washed with liquid soap and abundant water, then air-dried.
Male Response Assay

I measured the duration of searching behaviour of males exposed to the pheromone extracts as a measure of the activity and attractiveness of the sex pheromone produced by females of each treatment group and species. In previous studies males showed extensive activity on filter papers treated with silk extract from females (with changes in intensity and duration accordingly to female mating status and age), but not on methanol controls (Perampaladas et al., 2008; Stoltz et al., 2007). I scored male searching behaviour as total male movement in a 60 min trial when males were placed on filter paper treated with the experimental silk extract (sex pheromone + methanol) or methanol alone (control) placed inside glass petri plates (90 mm) according to the methodology used by Stoltz et al. (2007), Perampaladas et al. (2008), and Jerhot et al. (2010). For each trial, 0.2 ml of silk extract or methanol was added to a fresh disk of filter paper using a pipette, and the filter was allowed to air-dry for 5 min. Males were gently placed on the filter paper and the glass lid of the arena replaced. Trials were conducted between 10:00 and 20:00 hrs, and recorded on digital video using Panasonic WV BP330 low-light cameras and Navitar 7000 macro-zoom lenses. As both species are nocturnal, all trials were performed during the dark phase under red lights. After each trial, the filter paper was discarded and petri plates were washed with water and ethanol, and allowed to air-dry before reuse.

Each male (\(N = 50\) L. hasselti; \(N = 47\) L. hesperus) was exposed to the extract from a single experimental female and to a methanol control in random order with a 24 h interval between trials. Roughly half of the males were exposed first to methanol (L. hasselti, \(N = 25\); L. hesperus, \(N = 23\)) and half were exposed first to the extract (L. hasselti, \(N = 25\); L.
hesperus, \( N = 24 \)). Each female (\( L. \) hasselti, \( N = 50; L. \) hesperus, \( N = 47 \)) was used as a silk source only once.

Statistical Analysis

Analyses were completed in IBM SPSS version 20 (SPSS Inc., Chicago, IL, U.S.A.). Male activity levels and silk mass were not normally distributed in some of our treatment categories for both species (Shapiro–Wilk tests of normality, Table 3-1), and data transformations were unsuccessful. I thus used statistical tests for which I could specify the data distribution (generalized linear models, GLM, or generalized linear mixed models, GLMM) or nonparametric tests (Wilcoxon, Spearman rank correlations) to analyse the data within each species.

Female mass and silk production

I first tested whether my diet treatment affected female mass and silk mass using GLM (treatment = fixed factor; silk mass and female mass = dependent variables). I then examined whether silk mass was predicted by the mass of the female producing it, female’s diet treatment, or some interaction between these factors (GLMs: silk mass = dependent variable, female diet = fixed factor, female mass = covariate, and a diet*mass interaction term). Since female mass predicted silk mass in both species, I then describe this relationship directly using Spearman rank correlation.
Male activity assays

Before analyzing male activity results, it was necessary to first confirm the efficacy of my bioassay (i.e. that males respond significantly more to pheromone extracts than to methanol controls). Males of both species moved significantly more on silk extracts than on methanol-only controls in both diet treatments, showing they detected and responded to pheromones produced by females (Wilcoxon two-sample test: *L. hesperus*: unfed females: \( W = 190, P = 0.039 \); fed females: \( W = 273, P = 0.003 \); *L. hasselti*: unfed females: \( W = 262, P = 0.007 \); fed females: \( W = 291, P < 0.001 \); see Results, Table 3-1).

I analyzed variation in male sexual responses to extracts as a function of female diet treatment using GLMMs (e.g. Bolker et al., 2009) with a gamma distribution and log link since data were right-skewed. My initial model examined whether male activity (repeated measure, assessed on a control and one web extract treatment) was explained by five fixed factors: treatment (extract from fed or unfed female), female mass, silk mass, and two-way interactions between treatment and female mass or silk mass. The initial model also included the sequence of presentation of stimuli as a random factor, which allowed us to test whether there were any carryover effects arising from our design. For both species, the initial analysis indicated that the sequence of stimulus presentation did not affect male responses (random effect covariance = 0), so sequence was dropped from the model. I then used backward stepwise procedures combined with corrected Akaike Information Criterion (AICc) values to determine the best model to explain our data within each species. Fixed factors were dropped from the model in descending order according to \( P \) value. I report only the model with the lowest AICc (Appendix 3).
Results

Female Mass and Silk Production

The diet treatment affected female mass and silk production in both species (Table 3-1). Fed females weighed more (GLMs: *L. hesperus*: Wald $\chi^2 = 150.012$, $P < 0.0001$; *L. hasselti*: Wald $\chi^2 = 58.39$, $P < 0.0001$) and produced more silk (*L. hesperus*: Wald $\chi^2 = 9.27$, $P = 0.002$; *L. hasselti*: Wald $\chi^2 = 43.84$, $P < 0.0001$) than unfed females in both species (Table 3-1, Fig. 3-1). The effect of diet was dramatic, with female body mass decreasing by approximately 60% in both species (Table 3-1) when food was withheld. Similarly, silk production decreased by 48% in *L. hesperus* (consistent with Blackledge & Zevenbergen, 2007), and the decrease in silk production by *L. hasselti* females (67%) was even more substantial (Table 3-1).

There were positive correlations between female mass and silk mass (replicating Blackledge & Zevenbergen, 2007) in both species (Spearman rank correlation: *L. hesperus*: $r_S = 0.524$, $P < 0.001$; *L. hasselti*: $r_S = 0.732$, $P < 0.001$). Since this correlation could be a spurious result of the silk mass differences between our diet groups (even if the two factors are not strongly correlated within each diet group), I asked whether silk mass was more strongly predicted by diet, female mass, or some interaction between the two. In *L. hesperus*, the GLMs including these variables (likelihood ratio: $\chi^2_3 = 8.71$, $P = 0.005$) suggested that diet treatment (Wald $\chi^2_1 = 5.40$, $P = 0.020$) was the strongest predictor of silk mass, with female mass (Wald $\chi^2_1 = 3.18$, $P = 0.074$) mainly related to silk production via a diet*female mass interaction effect (Wald $\chi^2_1 = 4.12$, $P = 0.041$), which arose because silk and female mass were positively correlated only for unfed females ($r_S = 0.61$, $P = 0.003$). For *L. hasselti*,...
the model (likelihood ratio: $\chi^2_3 = 36.7, P < 0.001$) suggested that female mass (Wald $\chi^2_1 = 3.89, P = 0.049$) was a stronger predictor of silk mass than was treatment (Wald $\chi^2_1 = 0.480, P = 0.489$) or the interaction between these two (Wald $\chi^2_1 = 0.89, P = 0.767$).

Male Activity Assays

*Latrodectus hesperus*, but not *L. hasselti*, males responded strongly to differences in female diet (Fig. 3-2). *Latrodectus hesperus* males were more than three times more active on silk extracts of fed females than on silk extracts of unfed females (Table 3-1, Fig. 3-2). The best model ($F_{3,89} = 3.795, P = 0.013$) for variation in the activity of male *L. hesperus* included a significant effect of female diet treatment ($F_{1,89} = 11.087, P = 0.001$) and a nonsignificant interaction term between diet treatment and silk mass ($F_{2,89} = 2.980, P = 0.056$). None of the candidate GLMMs included significant main effects of silk mass or female mass, nor were there significant effects of the diet treatment*female mass interaction term in any candidate models.

In contrast, *L. hasselti* males had similar activity levels regardless of female diet (Table 3-1, Fig. 3-2). None of the candidate GLMMs were significant. While the full model had the lowest AICc, this model was not significant ($F_{5,86} = 0.550, P = 0.738$), and none of the fixed factors were significant predictors of male activity (all $P > 0.20$). Thus, male searching activity did not vary with silk mass produced by females or with female mass (see Fig. 3-3), nor were there any interactions of these factors with treatment (all $P > 0.1$).
Discussion

Here I show that information about diet is available in the chemical components or cues produced by female *L. hesperus* spiders, in this case, in the silk of the web. Male preference for well-fed females was not a correlated effect of the changes in the amount of silk produced as diet changed, as male response was not directly correlated with silk mass. Females apparently regulate silk and pheromone production independently, and changes in male response are most likely due to a decreased concentration of sex pheromone in the silk of poorly fed females. These results show that female response to food limitation includes changes in reproductive as well as foraging traits, and that males can detect these changes. I also show that diet causes substantial variation (40–60%) in silk production by females, confirming a previous study in one species (*L. hesperus*, Blackledge & Zevenbergen, 2007) and showing this effect for the first time in a congener (*L. hasselti*). Despite these substantial changes with diet in both species, males responded to information about diet only in the species at risk of precopulatory sexual cannibalism from poorly fed females (*L. hesperus*). This suggests that avoiding sexual cannibals could drive male mate preferences more strongly than seeking high-fecundity females in these spiders. More generally, although males may vary in response and preferences, it is clear that chemical communication can afford males considerable phenotypic information about females before they invest heavily in courtship, significantly reducing costs of male choice.

In my study, males of *L. hesperus* responded differentially to chemicals extracted from the silk of females on two different diets. Thus, there are apparently chemical cues in the silk that directly relate to the female’s recent feeding history, and this information is
detectable by males, indicating some qualitative or quantitative difference in pheromones produced by females on different diets. In other invertebrates, restricted feeding decreases the amount (but not the type) of pheromone produced. For example, in moths, sugar-deprived adult females produce fewer sex pheromone intermediates than females on full diets, but there is no change in chemical composition (Foster & Johnson, 2010), and male attraction to low-diet females decreases (Foster & Johnson, 2011; also see Harari et al., 2011). Similarly, female *L. hesperus* could be producing a lower quantity of pheromone when food deprived, just as they produce less silk. Such changes suggest that either silk (Pasquet et al., 1999) or pheromones are expensive to produce (see Gaskett, 2007; Harari et al., 2011; Jerhot et al., 2010), or that triggering male courtship when poorly fed is costly for females (e.g. Herberstein et al., 2002). In any case, our manipulations show that pheromones are honest signals of female feeding history in *L. hesperus*.

Although changes in diet triggered changes in silk production as well as differences in male response in *L. hesperus*, there was no direct link between silk mass and the activity or attractiveness of the sex pheromone as would be expected if females produce some consistent amount of pheromone per unit silk produced. Rather, pheromone production and silk production are apparently uncoupled, perhaps because females incorporate pheromone in only certain parts of the web, or only to a particular volume/concentration that is determined by nutritional condition. This is consistent with observations that changes in female condition result not just in changes in total web volume, but also in characteristics of the silk itself, and the amount invested in particular parts of the web (Blackledge & Zevenbergen, 2007; Zevenbergen et al., 2008).
While extracts from the silk of poorly fed females elicited a 42% depression in sexual responses in *L. hesperus*, *L. hasselti* males had high responses regardless of the diet or the mass of females that provided the stimulus (Table 3-1, Fig. 3-2), despite my use of the same methods and almost identical sample sizes. This is not because *L. hasselti* males are not choosy at all. It has been shown elsewhere that *L. hasselti* males distinguish virgins from nonvirgins based on airborne pheromones (choice in the field, Andrade & Kasumovic, 2005) and contact pheromones (preferences in the laboratory, Stoltz et al., 2007). Rather, this result suggests that, in contrast to *L. hesperus*, either (1) pheromone production by female *L. hasselti* does not change with diet, or (2) males can detect differences between females but this does not affect their response.

*Latrodectus hasselti* females might actively maintain sex pheromone production even under starvation because it can elicit an additional food source (i.e. courting males); however, there is little evidence for this type of deceptive signalling (e.g. Lelito & Brown, 2006). Moreover, since most pheromones have metabolic byproducts as precursors (Schulz, 2013; Wyatt, 2003), and a critical component of *L. hasselti*’s contact pheromone is a serine derivative (Jerhot et al., 2010), it would be surprising for hungry females to produce the same volume of pheromones as females with a more balanced energy budget. If anything, my results suggest that the shift in energy use when food was withheld was even more substantial in *L. hasselti* (67% reduction in silk) than in *L. hesperus* (48% reduction in silk). Thus it seems most likely that fed and unfed females differ in the concentration of the pheromone they produce in both species, but only males of *L. hesperus* are responding to this information.
*Latrodectus hasselti* males may not discriminate among females based on diet because there is no selective pressure to avoid females that are more likely to consume them. In contrast to *L. hesperus*, cannibalism does not prevent sperm transfer in *L. hasselti*, cannibalized males have higher paternity than those that survive, and in fact offer themselves to females during mating (Andrade, 1998, 1996; Forster, 1992). However, since diet also reflects female fecundity, even if cannibalism is not a risk, this leaves the question of why *L. hasselti* males do not have preferences for larger, well-fed females. The absence of preferences for these females may arise from the same factors that have led to high investment in one mating by males of this species (Andrade, 2003; Fromhage & Schneider, 2005). First, males that forgo mating with a smaller female may perish before finding a larger female (Andrade, 2003), and second, intense intermale sperm competition (e.g. Fromhage & Schneider, 2005) may mean that the main choice criterion is that the female is a virgin (Andrade & Kasumovic, 2005; Snow & Andrade, 2005; Snow et al., 2006). A similar effect is seen in *Argiope bruennichi*, a web-building spider where larger females are more fecund (Schneider et al., 2006), but monogynous males benefit when they are killed by females during copulation (Schneider & Elgar, 2001). Here, males do not preferentially seek out larger females in the field or laboratory, although they do have a strong preference for virgin over mated females (Schulte et al., 2010). This is in strong contrast to *L. hesperus*, in which male preference is strong for both virgin and well-fed females in the field (MacLeod & Andrade, 2014), as well as in my laboratory manipulation.

I conclude that the risk of sexual cannibalism, and by extension, any aspect of female phenotype that can significantly reduce mating success, may thus impose stronger selection on male preferences than variation in female fecundity. Although it is challenging to test this
hypothesis for the maintenance of male mate preferences, work on cannibalistic mantids suggests support. Lelito and Brown (2006, 2008) disentangled female size/mass (correlated with fecundity) from satiation level (correlated with cannibalism risk, Maxwell et al., 2010b) and showed that satiated females attracted more males than similarly sized food-deprived females, suggesting that male preferences are focused on decreasing cannibalism risk (Lelito & Brown, 2006, 2008; and see Brown et al., 2012). Similarly, my results showing differences in the dynamics of male preferences within species suggest that pursuing comparative tests of this hypothesis is warranted. *Latrodectus hasselti* and *L. hesperus* are congeners with conserved morphology, life history (Garb et al., 2004), and apparently, female responses to variation in diet (Fig. 3-1). The divergence in male preferences shown here would be predicted if cannibalism drives choice more strongly than fecundity. This genus, and others with comparable variation in mating systems and risk of cannibalism (e.g. Schneider & Andrade, 2011) may provide the ideal opportunity to use the comparative method (Harvey & Pagel, 1991) to test the hypothesis that the risk of a poor mate rather than the benefit of a good mate can drive the evolution of male mate preferences.

Acknowledgments. I thank E. C. MacLeod and M. M. Kasumovic for collecting and providing spiders from California and Australia and Andrade lab undergraduates and lab manager A. Baskaran for help rearing spiders. I am grateful to M. Modanu, E. C. MacLeod and an anonymous referee for comments on the manuscript. This study was financially supported by the Comision Sectorial de Investigacion Cientifica and the Instituto de Investigaciones Cientificas Clemente Estable (Uruguay, to L.B.) and the Natural Sciences and Engineering Research Council of Canada (Discovery grant), Canadian Foundation for Innovation and Research & Innovation Ontario (infrastructure grants) and Canada Research Chairs program (to M.C.B.A.). This study was conducted in accordance with animal care guidelines at the University of Toronto.
References


Table 3-1

Mean ± SE and normality tests (Shapiro–Wilk, W) for female mass, mass of silk produced and activity level of males exposed to methanol extracts from female’s silk for two species of *Latrodectus* spiders in which females were well-fed (fed) or food deprived (unfed) for 4 weeks

<table>
<thead>
<tr>
<th>Variable</th>
<th>Species</th>
<th>Fed</th>
<th>Unfed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female mass (mg)</td>
<td><em>Hesperus</em></td>
<td>358.59±16.049</td>
<td>135.16±7.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>W</em>₂₅=0.981</td>
<td><em>W</em>₂₂=0.980,</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>=0.913</td>
<td><em>P</em>=0.911</td>
</tr>
<tr>
<td></td>
<td><em>Hasselti</em></td>
<td>210.89±17.58</td>
<td>82.74±5.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>W</em>₂₁=0.918</td>
<td><em>W</em>₂₅=0.920</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>=0.079</td>
<td><em>P</em>=0.052</td>
</tr>
<tr>
<td>Silk mass (µg)</td>
<td><em>Hesperus</em></td>
<td>1.59±0.21</td>
<td>0.82±0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>W</em>₂₅=0.921</td>
<td><em>W</em>₂₂=0.798</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>=0.055</td>
<td><em>P</em>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td><em>Hasselti</em></td>
<td>1.26±0.14</td>
<td>0.42±0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>W</em>₂₁=0.783</td>
<td><em>W</em>₂₅=0.888</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>&lt;0.001</td>
<td><em>P</em>=0.010</td>
</tr>
<tr>
<td>Male activity (s)*</td>
<td><em>Hesperus</em></td>
<td>1421.36±194.01</td>
<td>830.59±143.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>W</em>₂₅=0.902</td>
<td><em>W</em>₂₂=0.857</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>=0.02</td>
<td><em>P</em>=0.005</td>
</tr>
<tr>
<td></td>
<td><em>Hasselti</em></td>
<td>1152.14±197.361</td>
<td>1049.16±161.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>W</em>₂₁=0.918</td>
<td><em>W</em>₂₅=0.920</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>=0.079</td>
<td><em>P</em>=0.052</td>
</tr>
<tr>
<td></td>
<td><strong>Control:</strong></td>
<td><strong>655.04±96.92</strong></td>
<td><strong>W*₄₇=0.819</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em><em>P</em>&lt;0.001</em>*</td>
<td><em><em>P</em>&lt;0.001</em>*</td>
</tr>
<tr>
<td></td>
<td><strong>Control:</strong></td>
<td><strong>557.44±79.89</strong></td>
<td><strong>W*₃₀=0.784</strong></td>
</tr>
</tbody>
</table>

*For this repeated measures design, tests for treatment effects on activity examine differences between controls and experimental treatments for each male (see Fig. 3-2); these global averages are provided for information only.*
**Figure 3-1.** Relationship between mass of silk (mg) produced by (a) *L. hasselti* and (b) *L. hesperus* females over a 4-day period as a function of the female’s mass (mg) and diet treatment (well fed: ◊; unfed: ■) in the preceding 4 weeks.

**Figure 3-2.** Mean ± SE difference in the time that *L. hasselti* and *L. hesperus* males spent searching on filter papers treated with silk extract of a conspecific female, which was either unfed (black bars) or fed (white bars) minus that male’s activity on methanol controls in 60 min trials. An asterisk indicates a significant difference between groups.

**Figure 3-3.** Relationship between male searching activity in response to extractions from silk of conspecific females and the mass of silk extracted as stimulus. All the silk produced by (a) *L. hasselti* and (b) *L. hesperus* females over a 4-day period after two diet treatments (well fed: ◊; unfed: ■) was extracted.
Figure 3-1
Figure 3-3
Copyright Acknowledgements
This chapter was published in *Animal Behaviour* (Luciana Baruffaldi & Maydianne CB Andrade. “Contact pheromones mediate male preference in black widow spiders: avoidance of hungry sexual cannibals?” *Animal Behaviour*. 102, 25-32) and is reprinted with permission of the journal.
Chapter 4

Is immature mating detrimental for females? An experimental study in redback spiders

Abstract

In a novel mating tactic recently described in *Latrodectus hasselti*, males mount juvenile females in their final instar and inseminate them by tearing their exoskeleton (‘immature-mating’), after which females moult and produce spiderlings. This is apparently adaptive for males, but may be maladaptive for females since there was little overt evidence of choice. Moreover, in our preliminary observations, some immature-mated (IM) *L. hasselti* females leaked haemolymph at the site of the exoskeletal tear. Here I assess effects of immature-mating on female mating tactics and reproduction to examine direct fitness consequences for females. In laboratory pairings, I confirm that males do not court immatures, in strong contrast to their prolonged courtship of adults. However, I find that immature females show elevated behavioural responses to male approaches and mounting attempts relative to adult females approached by courting males. Nevertheless, repeated copulations were more common in matings with immatures, suggested that apparent conflict over copulations may be a mechanism of choice. I predicted that, if immature mating is likely to be adaptive or neutral to females, IM females may not produce sex pheromones as adults, mirroring the behaviour of adult-mated (AM) females. Bioassays of male responses to pheromone extracts suggested IM females are not soliciting additional mates as they apparently do not produce
sex pheromones at the levels found in virgin adult females. Finally, under laboratory conditions, IM females had similar longevity, fertility and fecundity as AM females, suggesting there is no direct reproductive cost for females. Thus, although, males tear the exoskeleton of females and do not perform courtship (a key trait favoured by adult females) I found no evidence of costly coercive mating by males. Rather, I argue that being mated as an immature could be beneficial for females which can avoid the risk of remaining unmated as adults, while minimizing energy expenditure for mate attraction.
Introduction

In many animals, females’ fitness may be affected by mating decisions that determine direct benefits or genetic qualities derived from the male with which they chose to mate, whereas males’ fitness increases with the number of copulations achieved with different females (Bonduriansky, 2001; Parker, 1979). Therefore, in general females are the choosy sex and males compete for access to females and paternity (reviewed in: Bonduriansky, 2001; Edward & Chapman, 2011). Sexual selection on males to access females has driven the evolution of a wide range of courtship behaviors (Parker, 1974), including a variety of vigorous sexual displays, ornamentations or gifts that may persuade females to accept male mating attempts (Andersson, 1994; Candolin, 2003; Eberhard, 1996). In some species however, intense sexual conflict over mating frequency has instead produced male “coercive tactics”, structural adaptations, and behaviours for mating while bypassing courtship and other forms of mating effort (Arnqvist & Rowe, 2005; Clutton-Brock & Parker, 1995; Chapman, 2006; Evans et al., 2003; McKinney et al., 1983; Shine et al., 2003). Coercive matings have been describe in the literature as copulations that decrease female fitness relative to forgoing mating (e.g., Arnqvist & Rowe, 2005; McClain & Pratt, 1999; McKinney et al., 1983; Thornhill, 1980), but may occur if males use physical means to force copulation, harass females sufficiently that resistance is more costly than mating, or punish females that will not mate (Clutton-Brock & Parker, 1995). For example, males may attempt to mate when females are eating, sleeping, or (in invertebrates) when the body cuticle is still soft immediately after a moult (Danielson-François et al., 2012) and females cannot repel males or defend themselves (Arnqvist & Rowe, 2005). Coercive males may use their strength, their
bodies, or specialized structures, like claspers, to retain and hold on to the female during coupling and thus force copulation, or may repeatedly attempt to mount and mate with females (harassment) instead of persuading females with courtship before and during mating (Arnqvist & Rowe, 2005; Chapman, 2006; Evans et al., 2003; McKinney et al., 1983; Peretti & Willemart, 2007; Thornhill, 1980). Consistent with theory (Clutton-Brock & Parker, 1995) coercive mating has been reported in species in which there is strong male-male competition for relatively few receptive females (e.g., seed bugs: McClain & Pratt, 1999; Drosophila, Dukas & Jongsma, 2012a,b; Seeley & Dukas, 2011; cave crickets, Conroy & Gray, 2014), the sex ratio is biased towards males (e.g., water striders, Arnqvist & Rowe, 2002; Rowe et al., 1994; waterfowl, Blum & Mednis, 1996), there is a high predation risk for courting males (e.g., guppies, Evans et al., 2003), and/or high costs associated with acquiring resources required to persuade females to mate (e.g., Panorpa scorpionflies, Thornhill, 1980). Although males of some species show almost exclusively coercive tactics (Bisazza et al., 2000), in other species, these tactics may also be adopted only in certain contexts or only by certain males.

Females may show distinct behaviours in response to coercive mating attempts, such as shaking their bodies, moving rapidly or even fighting with males before or during mating (Arnqvist & Rowe, 2002; Bisazza et al., 2000; McClain & Pratt 1999; McKinney & Stolen, 1982). These behaviours may represent female resistance to coercive males, suggesting that mating is more costly to females than is resistance (Arnqvist, 1989, 1992; Clutton-Brock & Parker, 1995; Chapman, 2006). Resistance may have negative impacts on female fitness and survivorship, because of increased energy expenditure and predation risk in addition to lost opportunities to find a more suitable male (Arnqvist & Rowe, 2002; Rowe et al., 1994;
Watson et al., 1998). On the other hand, resistance behaviours may instead (or also) be a mechanism used by females to evaluate male quality, as persistent or physically powerful males may be superior mates and this response may then represent classic female choice (Allen & Simmons, 1996; Eberhard, 2004; Thornhill, 1980; West-Eberhard, 2014). Thus, although females are predicted to resist coercive behaviours and matings, it is not possible to distinguish the function of female resistance behaviours simply by observation, and the logic of using such observations to suggest “coercive mating” is flawed. Rather, the critical question may be whether or not females suffer fitness deficits when they mate with “coercive” males (i.e., males that forgo courtship, Peretti & Cordoba-Aguilar, 2007; West-Eberhard, 2014).

Here, I ask whether a novel male mating tactic, recently described in the Australian redback spider, Latrodectus hasselti (Biaggio, 2007) may be coercive by examining how this tactic affects female reproductive fitness, behavioural responses to males during mating, and the pattern of females’ pheromone-mediated attractiveness after mating. In this unusual tactic, male L. hasselti mate with females in their final juvenile instar (sexually immature females) by tearing the exoskeleton covering their fully-developed, but otherwise concealed genitalia (‘immature mating’, Biaggio 2007, Fig. 1). Immature-mated females moult then produce fertilized eggs using sperm which is stored successfully through the moult. In L. hasselti the mating behaviour of adult females is well characterized and adult females show preferences for larger males, and high courtship effort (Snow & Andrade, 2005; Stoltz et al., 2008, 2009; Stoltz & Andrade, 2010). L. hasselti males most typically mate with adult females, but about 1/3 of immature females may be mated in the field (estimated across two field seasons, MCB Andrade, pers comm). In matings with adults, females ‘set the rules’ for
successful matings (Eberhard, 1996), and there is strong evidence for female choice based on prolonged (mean = 5hr, Forster 1995), costly (De Luca et al., 2015; Kasumovic et al., 2009) vibratory courtship by males (Stolz et al., 2008, 2009; Stoltz & Andrade, 2010). Females are often polyandrous (Andrade, 1996) and discriminate against males that attempt mating without sufficient courtship by killing them before mating is complete (premature cannibalism), and by mating more frequently with their rivals (Stolz et al., 2008, 2009; Stoltz & Andrade, 2010). Since these responses affect paternity (Snow & Andrade 2005), female’s behavioural responses impose selection on males to court, and this ‘rule’ may explain the prolonged courtship typical of matings with adults (Stoltz & Andrade, 2010).

In the only study to date of this immature mating behaviour, vibratory courtship was minimal when males mated with immature females, in contrast to typical matings with adults (Biaggio, 2007), but nevertheless, there was no premature cannibalism, and immature females were more likely than adult females to permit males to complete a full mating (Biaggio, 2007). The absence of persuasive courtship and typical female choice mechanisms (such as premature cannibalism, Stoltz & Andrade, 2010) suggests these matings may be coercive. Moreover, in my preliminary trials, at least some immature-mated females showed haemolymph bleeding at the area cut open by males. However, it is not yet clear whether females show resistance to male mating attempts since there has been no detailed analysis of male or female mating behaviours to date (see Biaggio, 2007).

Here I compare the inter-sexual behaviors, female fitness, and pheromone-mediated sexual attractiveness of mated females after copulations as immatures or adults to test whether immature mating is coercive. First, I examine whether immature females are more
likely than adult females to engage in behaviours that reduce the likelihood of males mating successfully (‘female resistance’). If immature mating is coercive, then females should exhibit behaviours that would deter male approach or mating attempts (Arnqvist & Rowe, 2002; McKinney & Stolen, 1982), regardless of whether this globally reduces mating frequency (conflict) or biases mating success to preferred mates (choice). Second, I test one prediction of the coercive mating tactic hypothesis; that immature-mated females will have lower direct fitness (measured as fecundity, fertility and post-mating survivorship) than females mated as adults. Previous work did not detect a fitness cost of mating for immature females, but this was based on a small sample size (n = 11) and did not include an assessment of survivorship, which is known to be affected by coercive mating in other taxa (Dukas & Jongsma, 2012a,b; Seeley & Dukas, 2011). I tested these predictions in a laboratory study in which I compared behavioural dynamics and outcomes of pairings between males and adult or immature females, then tracked female fecundity, fertility, and survival after mating as measurement of direct fitness.

Third, I investigate effects of immature mating on female mating tactics by inferring patterns of sex pheromone production of females following mating. The detection of chemical cues produced by females is essential for mate attraction and/or the initiation of courtship in widow spiders (and other invertebrates Gaskett, 2007; Johansson & Jones, 2007; Thomas, 2011). Sender-receiver coevolution should ensure the timing of sex pheromone production will, on average, honestly reflect variation in female receptivity (Harari et al., 2011; Thomas, 2011). Variation in the production of sex pheromones should thus track the fitness value of mate attraction and copulation for females. In L. hasselti, virgin adult females produce sex pheromones that attract males and trigger courtship (Andrade & Kasumovic,
2005; Jerhot et al., 2010). Females mated as adults cease sex pheromone production immediately after mating (Jerhot et al., 2010; Stoltz et al., 2007), and hence stop attracting males (Andrade & Kasumovic, 2005). If immature-mating is costly to females because they do not choose their first mate, they may continue to produce sex pheromones as adults to solicit additional matings, as is typical for newly-moulted virgin females (e.g., Dunkas & Jhogsma, 2012b). Alternatively, if immature mating is neutral or beneficial to females, then females mated as immatures may not produce sex pheromones after mating and moultng; mirroring adult-mated females. I test these predictions by assaying male responses to pheromone extracts from the silk of females (Jerhot et al., 2010) mated as adults or immatures compared to an age-matched group of adult unmated females. This study will allow inferences about whether immature mating is a form of coercive mating, and ultimately provide data contributing to a comparative analysis of the origin and maintenance of this behaviour across *Latrodectus*.

**Methods**

*Latrodectus* biology & natural history

*L. hasselti* is an extremely sexually dimorphic species with females hundreds of times heavier than males (female weight 123.13 mgr ± 37.45, male weight 3.18 mgr ± 1; Forster, 1995). Like other web-building spiders, females are sedentary, spending most of their life in one web. Males, on the other hand became nomadic when they mature, leaving their own web and searching for mates, using females’ chemical signals to find and discriminate among potential mates as a function of developmental stage and mating status (Andrade & Kasumovic, 2005; Stoltz et al., 2007). Males commence courtship when they come in contact
with the silk of a virgin female, triggered by a web-borne pheromone (an acylated serine derivative) that can be extracted from silk in methanol and is not present in the silk of adult females right after mating (Jerhot et al., 2010; Perampaladas et al., 2008; Stoltz et al., 2007). Courtship is a prolonged sequence of vibratory movements that begins on the web, and continues on the female’s abdomen before mating is attempted (5 hours after courtship begins, Forster 1992, 1995). Courtship vibrations are produced by males flexing and extending their legs and vibrating their abdomens, both of which are clearly visible to a trained observer.

The adult female’s external genitalia are a hardened, raised area of cuticle (epigynum) within which are two genital openings, each of which is connected to one of two sperm storage organs (spermathecae) from which sperm are taken in roughly equal proportions at fertilization (Snow & Andrade, 2005). To maximize paternity, males must inseminate both spermathecae, and this is accomplished only if they copulate twice, inserting one of their 2 pedipalps (organs than carry and transfer sperm) into one genital opening at each copulation (one ‘complete’ mating). The two palpal insertions are separated by a period of additional courtship on the web (~20 min. Forster, 1992). Males frequently leave behind a sperm plug (the broken tip of their copulatory organ), which does not necessarily prevent male or female remating, but is linked to first-male sperm precedence if they deposit the plug successfully (Snow & Andrade, 2005; Snow et al., 2006).

Receptive females are quiescent (Andrade, 1996) and accept male mounting and mating attempts with relatively little movement. In contrast, females are sometimes agitated during pairing, periodically moving about the web and/or contacting males with their legs.
during courtship or mounting, and in these cases male mating success is typically lower (Andrade, 1996) or copulation duration may be reduced (e.g., *L. tredecimguttatus*: Neumann & Schneider, 2011).

Genital development

The female’s final juvenile instar is approximately 11 days in duration (11.3 ± 3.2, Biaggio, 2007) during which time females have no external genital opening, and initially, there are no recognizable internal genitalia. As the final moult approaches, however, the proportion of females with partially or fully developed genitalia increases so that by day 7, approximately 2/3 of females have fully developed spermathecae, and all females are fully developed by day 8 (Biaggio, 2007) although there is no external genital opening (Fig. 4-1).

Experimental Spiders

Juvenile *Latrodectus hasselti* (Thorell 1870) were reared from an outbred laboratory population started with mated females collected in Sydney, New South Wales, Australia in January 2010. Spiders were reared with siblings in communal cages (8 x 8 x 12 cm Amac Plastics, Ltd.) for the first 2 instars then moved to individual 5 x 5 x 7cm clear plastic containers (Amac Plastics, Ltd.) to ensure that they had not mated at the time of the trials. Spiderlings and males were fed twice weekly with *Drosophila sp*. fruit flies and females were fed with one cricket (*Acheta domesticus*, fed a protein-rich diet of kitten chow mixed with fish flakes) per week from approximately the fifth instar until they became adults. All spiders were reared in a temperature-controlled room at 25° C (12 hrs light, 12 hrs dark).
Experimental females

Immature females were identified by examination of the region where the genital plate (epiygynum) develops under the microscope. In immature females, the copulatory openings are always closed (Fig. 4-1A), but the entire area protrudes as the insemination tubules and the sperm storage organs (spermathecae) develop under their exoskeleton (Biaggio, 2007). Immature females with a protuberant epigynal area were randomly assigned to 3 experimental groups: (1) Immature-mated (IM) females were paired with adult males during their immature instar, typically between 2 to 4 days before their final moult to adulthood (determined post-hoc), (2) Adult-mated females (AM) females were paired with males 3 to 10 days after their final adult moult, and (3) Virgin females (V) were never exposed to males and so remained unmated.

Mating trials

Females assigned to the IM and AM experimental groups were placed in mating arenas for 48 h (35×30×15 cm) to construct webs on metal frames prior to the introduction of males. Trials occurred during the dark phase (L. hasselti are nocturnal) and commenced when males were placed on a web and were terminated after mating occurred or after 8 hours. Digital videos of trials were recorded using low-lux red-light illumination, and Panasonic low-light black and white cameras (WV BP330) with macro zoom lenses (Navitar Macro-Zoom 7000). Inter-sexual interactions from the 19 successful mating trials for each group were analyzed.

I quantified behaviours of males and females that reflect mating progress from male approach through copulation (described in detail in: Forster, 1995; Stoltz et al., 2009), as well
as measures of pairing success such as the occurrence and frequency of copulation and copulation duration (which can affect paternity, Snow & Andrade, 2005, Table 4-1, 4-2). Although courtship behaviours and progress will inevitably be the result of interactions between the sexes, I categorized these in terms of which sex initiated the behaviour (males: Table 4-1; females: Table 4-2).

Direct fitness effects of mating

Females mated in the IM and AM treatments were returned to their rearing cages, fed as before, then monitored three times per week to record the production of egg sacs and the timing of death. Eggs sacs were removed from the female’s cage and individually housed in plastic cages. Immediately after spiderlings emerged from the sac, the cage was placed in the freezer to avoid sibling cannibalism and allow later counting of spiderlings and eggs. The number of spiderlings and eggs produced in each first egg sac was counted for all experimental females (adult female reproductive output per egg sac does not vary significantly across the first 15 sacs produced in the lab, Andrade & Banta, 2002). Fecundity was calculated as the total number of eggs produced (= spiderlings + unhatched eggs inside the sac), while fertility was the number of spiderlings that hatched divided by fecundity.

Silk collection and pheromone extraction

Between 1 and 2 weeks after moulting to adulthood, but before the deposition of any egg sacs, females from the 3 experimental groups were placed in silk-collection arenas (consisting of clean, paired, inverted U-shaped stainless-steel wires supported in a plastic block surrounded by a water bath) and each was allowed to build a web for 4 days (Chapter
Females were removed and the silk produced was harvested using glass Pasteur pipettes (line glass 9”) and placed in 2 ml auto-sample Teflon cup glass vials (National Scientific). Sex pheromone associated with the silk was extracted by submerging the silk in 0.15 ml of methanol (HPLC, 99.9%, Fisher Chemicals) for 24 h. Between collections, all components of the arenas were washed with liquid soap and abundant water, then air-dried. New pipettes and vials were used for each collection.

Sex pheromone bioassay

The presence and attractiveness of the sex pheromone produced by females of each experimental group was measured by the duration of male searching behaviour on pheromone extracts using well-worked-out methodology (Baruffaldi & Andrade, 2015; Jerhot et al., 2010; Perampaladas et al., 2008; Stoltz et al., 2007). Male courtship and searching behavior was scored in a 60 minute trial where males were placed on filter paper (Fisher Scientific, 09-795C) treated with the experimental silk extract (extract + methanol) or methanol alone (control, C) placed inside glass petri plates (90 mm). For each trial, 0.15 ml of silk extract or methanol was added to a fresh disc of filter paper using a pipette, and the filter was allowed to air-dry for 5 minutes. Naïve virgin males were gently placed on the filter paper and the glass lid of the arena replaced. Trials were conducted between 10am and 8pm, and recorded on digital video using Panasonic WV BP330 low-light cameras and Navitar 7000 macro-zoom lenses. All trials were performed during the dark phase under red lights, and after each trial, the filter paper was discarded and petri plates were washed with water and ethanol, and allowed to air-dry before reuse. Each female was used as a silk source only once, and each male was used only once.
Statistical analysis

Analyses were completed in IBM SPSS Version 22 (SPSS Inc., Chicago, IL, U.S.A.). Some female and male behavioural data were not normally distributed in some of our behavioural categories (Shapiro-Wilk tests of normality), and data transformations were unsuccessful. The same was observed with some of our female fitness and male activity data. I thus used General Linear Models (GLM) to analyze normal data, Generalized Linear Models (GLMS) with Gamma log link error distributions for analyzing durations, and GLMS with Poisson log linear error distributions for analyzing occurrence data (counts). Post-hoc power analyses were completed using G*Power for multiple linear regressions (Faul et al., 2007).

Here I report mating rates, but analyze in detail only trials in which males successfully copulated with females at least once, since estimating the developmental state of immature females is inexact, and failed pairings may represent cases where females’ genitalia were insufficiently developed to allow mating (e.g., Biaggio, 2007).

Pre-copulatory interactions and Mating outcomes

I first tested whether there were differences in the frequency ($\chi^2$ test) of behaviors performed by males or females in IM compared to AM matings. Then I asked if female treatment (IM or AM) affected male or female behaviour using GLMS or GLM (treatment = fixed factor; behaviours = dependent variables). Since our observations suggested that females used an abdomen scraping behaviour (Table 4-2) to attempt to remove mounted males, I also tested whether the frequency of abdomen scrapes increased with the time prior to the male’s first mating (using GLMS).
Direct fitness effects of immature mating

I analyzed whether female mating treatment was related to variation in females’ longevity, latency to deposit their first egg sac, or fecundity (all normal data, Shapiro-Wilk p > 0.05) using separate GLM’s. The effect of mating treatment on female fertility (non-normal, Shapiro-Wilk p < 0.001) was examined using GLMS. All models included female body size as a covariate.

Sex pheromone bioassay

I tested for an effect of our treatment on male activity using a GLMS including treatment (Control, V, IM or AM) as a fixed factor and male searching behavior as a dependent variable. Post-hoc pair-wise comparisons among the different treatments were used to determine the efficacy of our bioassay (i.e., that males responded significantly more to pheromone extracts than to methanol controls) and differences between treatments.

Results

Pre-copulatory interactions

Most males (79%, n = 19) approached immature females without any vibratory courtship on the web, although courtship was evident in every mating trial with adult females (n = 19; $\chi^2 = 4.471, p = 0.034$). Nevertheless, the time from the start of trials until the first mounting attempt and first copulation was similar for both treatment groups (Table 4-1). For males mating with adults, this time was spent initially courting on the web, and then in a period when males alternate courtship on the web with courtship while mounted on the female’s
body (also see Forster, 1995). Males mating with adults mounted females repeatedly (19 times on average) during this period, prior to their first mating attempt (Table 4-1). In comparison, males paired with immature females mounted females only 7 times on average before their first palpal insertion (Table 4-1). While males were mounted, prior to the first palpal insertion, females sometimes swept their back legs across the part of their abdomen on which males were standing (= “abdomen scraping”). This scraping behaviour sometimes resulted in males quitting the abdomen and returning to the web before making another mounting attempt. In other cases, however, the male responded by remaining on the abdomen but moving away from the female’s genitalia and towards the spinnerets. Immature females were three times more likely than adult females to scrape at mounted males on average (GLMS: Wald-χ² = 22.155 p < 0.001, Table 4-2). This difference is even more pronounced when normalized to the number of mounts achieved by males (AM: females scraped in 3.8% of mounts; IM: females scraped in 43% of mounts). Moreover, scraping behaviour increased with interaction time (GLMS Likelihood ratio χ² = 32.325, df =3, p <0.001, model includes male mass as a non-significant covariate), but not in both treatments. IM females scraped at males more frequently when males spent longer attempting their first mating (latency to 1st copulation, Table 4-1; GLMS Wald-χ² = 7.483, p = 0.006), but this was not the case in AM matings (GLMS Wald-χ² = 1.046, p = 0.306, Fig. 4-2). Progress toward mating was also affected by female movement, as males retreated from females and ceased their own movement if females became mobile. Immature females spent about 22 minutes moving about the web during trials compared to only 8 minutes for adult females (GLMS: Wald-χ² = 9.081 p = 0.003) (Table 4-2). Thus, while the latency to the first copulation for adult females was mainly taken up with male courtship, for immature females, this latency was mainly the
result of female-caused delays to male mounting and mating attempts (abdomen scraping, movement, Table 4-1, 4-2).

Mating outcomes

While 80% of males successfully mate with adult females in my laboratory trials (Chapter 2), only 65% of males paired with immature females mated successfully ($\chi^2 = 4.97, p = 0.025$). However, while 26% of the males mating with adults were killed during their first insertion, all males paired with immatures survived the first insertion ($n = 19, \chi^2 = 5.758, p = 0.016$, Table 4-2). Moreover, males paired with immature females achieved two palpal insertions in almost every mating (95%, $n = 19$) compared to only about 70% of males paired with adult females ($n = 19, \chi^2 = 4.358, p = 0.036$, and see Biaggio, 2007).

In matings with adults, males performed the somersault behavior in every copulation, and only 47% survived mating ($n = 19$), in comparison, only one male somersaulted when mating with an immature female ($n = 19, \chi^2 = 34.2, p < 0.0001$) and all males that mated immatures survived ($n = 19, \chi^2 = 13.571, p = 0.0002$). Whereas every surviving male mated to an adult female returned to the web after the first palpal insertion and resumed courtship prior to attempting a second copulation, 58% ($n = 19$) of males paired with immature females did not dismount the female prior to the second insertion ($\chi^2 = 8.972, p = 0.0027$). Thus, both the number of mounts prior to a second insertion and the latency to remount after the first insertion were lower for IM males than for AM males (Table 4-1). Males mated to adults also moved on and off the female’s abdomen more frequently between insertions (Table 4-2). This is likely why second palpal insertions tended to be achieved much more quickly by males mating immatures (~19 min) compared to males mating adults (~32 min, Table 4-1).
In addition, second insertions lasted longer than second insertions of males paired with adults (Table 4-1).

**Direct fitness effects of immature mating for females**

I found that IM females (N=22) have similar longevity (GLM: F = 0.051 p = 0.822) and latency to deposit their first egg sac as AM females (N=17) (GLM: F = 1.270 p = 0.267) (Fig. 4-3). Moreover, IM females (N=21) have similar fertility (GLMS: Wald-χ² = 0.098 p = 0.755) as AM females (N=18, Fig. 4-3). While fecundity of AM females tended to be slightly higher, than IM females, this difference was not significant (GLM: F = 3.991 p = 0.054). Power for these analyses was 0.65 assuming a medium effect size (range: small effect, power = 0.13; large effect, power = 0.95).

**Sex pheromone bioassay**

As expected, males spent much of their time searching and courting on the silk extract of virgin females (almost ½ of the trial), significantly more than the time spent moving on the solvent-only control (GLMS: Wald-χ² = 11.813, p = 0.001, Fig. 4-4). Male response to the silk of AM females was similar to the control (GLMS: Wald-χ² = 0.469 p = 0.493), consistent with the reported cessation of pheromone production by these females (Jerhot et al., 2010; Stolz et al., 2007). Similarly, there was no difference between male response to extracts from IM females compared to the control (Fig. 4-4, n = 19; GLMS: Wald-χ² = 1.230 p = 0.267), suggesting a similar lack of pheromone activity.
Discussion

In *Latrodectus hasselti*, the progress, inter-sexual behaviours, and outcome of matings of males with immature females (IM) are very different from matings with adults (AM). Males that mated with immatures showed no evidence of the continuous, persuasive, largely web-based courtship that is common before each copulation for males paired with adults (Forster, 1995). Rather, males approached immature females without signalling (also see Biaggio, 2007), mounted, then typically remained mounted until two copulations were achieved. In response, immature females showed a relatively high frequency of behaviours that are likely to delay mating (‘abdomen scrapes’ and movement, Table 4-2), and may have resulted in the decreased IM mating success observed here. Despite these female behaviours, males that mated with immatures were more successful than those that mated with adults in terms of the frequency with which they achieved two copulations (95% IM; 70% AM), which is likely mediated by a significant difference in the occurrence of cannibalism after the first copulation (‘premature cannibalism; 0% IM; 26% AM). Thus, neither males nor immature females behave as expected in matings with adults, where males approach females with prolonged courtship. Nevertheless, I did not find any evidence that being mated as an immature was detrimental for females in terms of their direct reproductive output or survival (Fig. 4-3). Similarly, I found no evidence of IM females advertising to other males as might be predicted if IM mating is detrimental because it circumvents female choice. If IM was costly, I expected females to produce attractive sex pheromones after their final moult, as they would if unmated (Stoltz et al., 2007). However, I found no evidence for this in my laboratory bioassay (Fig. 4-3). Thus, even though immature mating involves no apparent
courtship by males, ‘resistance’ behaviours from females, and breaching the female’s exoskeleton, I conclude that this behaviour shows little effect on females’ direct fitness. Moreover, given that *L. hasselti* females experience non-negligible risk of mating delays in nature (Andrade & MacLeod, 2015; Andrade & Kasumovic, 2005), selection for ensuring fertility may be strong (Kokko & Mappes, 2005) so maturing with stored sperm from immature mating may be adaptive for females. Thus I argue that immature mating is not coercive and ‘resistance’ behaviours may be better understood as mechanisms of choice.

When *Latrodectus hasselti* males are paired with adult females, the sequence of courtship behaviours is similar to that seen across most described species in the genus (Forster, 1995; Stoltz et al., 2008, 2009), and includes three phases. In the first phase the male produces vibrational signals on the web exclusively, the second phase involves males alternating periods of courtship on the web with mountings or contact with the female. Most males attempt copulation during a third phase, when they spend most of their time mounted on females, producing vibrations, and probing the epigynum with their palps (Forster, 1995; Stoltz et al., 2008, 2009). For males paired with immature females, this courtship progression was completely absent. Rather, males mounted immature females without producing vibrations and typically remained there (thus the number of mounts was much lower for IM males than for AM males, Table 4-1). In response to male mounting, while receptive adult females generally remain immobile, immature females more commonly attempted to remove males from their abdomen (‘abdomen scraping’), scraped more when males remained mounted for longer, and spent more than twice as much time moving about the web than did adults (Table 4-2). Similarly, after one copulation was achieved, IM males attempted to remain on the female’s abdomen until they achieved a second copulation despite the
continued ‘scraping’ behaviour of females (Table 4-2). This is in strong contrast to AM males that survive their first copulation, as these males return to the web prior to attempting a second copulation. The discrete nature of the two copulation attempts, separated by a period of additional courtship, facilitates choice by AM females with relatively little energy expenditure since female may allow one copulation but avoid a second either by killing or rebuffing the male during the dismount (Table 4-1, and see Stoltz et al., 2008, 2009). Thus prolonged mounts of IM females may constitute male harassment (Clutton-Brock & Parker, 1995), and scraping behaviour may be equivalent to the costly struggles of females attempting to remove mounted or mating males in other taxa (e.g., water striders, Watson et al., 1998). The cost of attempting to remove mounted males may thus constrain choice (e.g., Bisazza et al., 2001; Clutton-Brock & Parker, 1995). Choice by IM females may also be affected by a lack of information; vibratory courtship in AM matings apparently functions as an endurance test, with duration affecting female responses to males, including the likelihood of premature cannibalism, and the likelihood that a given male will achieve two copulations (Stoltz & Andrade, 2010; Stoltz et al., 2008, 2009). Thus in IM matings, males are not transmitting the same types of information to females, and the exercise of choice by females is likely to be more costly because other information is not present.

These significant differences between typical matings and IM matings are consistent with predictions from a coercive mating hypotheses. Although males are much smaller than females and have no apparent morphological specializations for coercion (see Parker, 1979; Smuts & Smuts, 1993), there are other examples of coercion despite female-biased size dimorphism. For example, female garter snakes (*Thamnophis sirtalis*, Shine, 1994) can prevent intromission by keeping their cloacae closed (Gillingham, 1987) but when coercive
males constrict around females, hypoxic stress leads to female cloacal gaping (a response usually interpreted as an antipredator adaptation, Greene, 1988) and thus mating access (Shine et al., 2003). Thus general features of anatomy or physiology of females, rather than specialized structures of males, can facilitate forcible copulation in some species. For example, in some species of arthropods males mate with recently moulted females, when the females’ soft exoskeleton makes it unlikely that they can resist copulations (Danielson-Francois et al., 2012; Seeley & Dunkas, 2011). In *Latrodectus*, the protuberant developing genitalia of immature females may facilitate males’ identification of the critical area and allow them to use their fangs to expose the copulatory openings without significant damage to the female. If pre-molt IM females were also unable to resist males due to the physiological changes associated with ecdysis, this may also make coercive mating likely. However, despite the proximity to moult (Biaggio, 2007), the frequent (sometimes successful) attempts of females to remove males from their abdomen (‘abdomen scraping’ Table 4-2) shows they are capable of effective resistance. Moreover, despite apparent male harassment and female scraping, there was no evidence that IM females tried to kill males, and in fact they were much more likely to cannibalize males when paired as adults (Table 4-1). This is striking since male harassment and their own resistance is likely to be costly for females (energetically, and by increasing predation risk and decreasing foraging opportunities). These costs may be particularly acute because IM females are preparing for the delicate moulting process, and physiological changes related to shedding the old cuticle may make it physically challenging for females to engage in such behaviour. Thus, despite female ‘resistance’ behaviours, there are reasons to question whether IM matings are in fact coercive.
Regardless of inter-sexual behavioural interactions, IM females should suffer some fitness deficit relative to AM females if matings are coercive. In my study, *L. hasselti* females mated as immatures showed no significant decrease in fertility, fecundity or longevity compared to adult-mated females (Fig. 4-3). Sample sizes in this study (two mating treatments, total n = 38) were comparable to those used in other laboratory experiments on *L. hasselti* that have demonstrated significant effects of diet and mating status on adult female longevity and reproductive output (e.g., Stoltz et al., 2010, three treatments, total n = 50). Moreover, no significant differences in fertility and fecundity measures were found for IM compared to AM females in Biaggio’s (2007) study, albeit at small sample sizes (two treatments, total n = 22), nor in 2 other *Latrodectus* species where immature mating has been studied *L. geometricus* (Baggio et al., in prep) and *L. hesperus* (Appendix 2). In contrast, in most studies that demonstrate coercive mating, there are severe repercussions for females’ direct fitness (large effect sizes). For example, in mating aggregations of garter snakes, the constriction behaviour of coercive males sometimes causes the suffocation death of resistant females (Shine et al., 2000). In several taxa, physical injuries to females occur during fights with males and these decrease female longevity (Stone, 1995). In others species, female egg production decreases substantially after coercive mating (likely due to the energy expended in resisting, Watson et al., 1998), or predation risk on resistant females increases during coercive attempts (Arnqvist & Rowe, 2002; Dukas & Jongsma, 2012a,b). There was no evidence of similar dramatic effects of IM mating in this study.

Despite my results, it is possible that IM-mated females could suffer direct fitness deficits in the field that are not evident in the laboratory. For example, my observation that
some IM females showed haemolymph bleeding near the genitalia immediately following IM matings might indicate that these matings sometimes provide a route for infection. This possibility that would have to be investigated in the field, but it seems likely that such effects would compromise longevity in the laboratory as well, where females are allowed to retain old prey carcasses (possible sources of infectious microbes) in their cages, as they do in their webs in the field. Another potential reason IM mating could be costly is because IM females do not cannibalize their mates (Table 4-2) and so would forgo any fitness benefits associated with cannibalism. Sexual cannibalism has been shown to accelerate the timing of egg sac production (Rabaneda-Bueno et al., 2008), and to increase offspring hatching success (Berning et al., 2012; Pruitt et al., 2014), condition or survival (Rabaneda-Bueno et al., 2008; Wu et al., 2013) in several spider species with low sexual size dimorphism. However, in species with strong female-biased size dimorphism, including *L. hasselti*, several studies suggest no direct effects on female fecundity or fertility (reviewed in Schneider & Andrade, 2011). These older studies typically examined only egg sac production and hatching rates, whereas recent work in a size-dimorphic web-builder showed that the offspring of cannibalistic females have increased longevity under food stress (starvation, Welke & Schneider, 2012). Nevertheless, such effects are unlikely to lead to strong deficits in IM female fitness for a number of reasons. First, even in the size-dimorphic species where cannibalism increased offspring survival, the median longevity of the unfed spiderlings of non-cannibalistic females was 4 weeks (Welke & Schneider, 2012, Fig. 4-3); a substantial period that is likely to be sufficient for spiderlings to establish their own webs and catch prey in the field. Second, *L. hasselti* females produce an average of 6 egg sacs in their lifetime in nature (Andrade & Banta, 2002), with each containing ~100 to 300 offspring (Forster, 1995).
which disperse by ballooning. For this r-selected species, small to moderate variation in
offspring number, hatching success, or survivorship under starvation is unlikely to have a
significant effect on female fitness. Moreover, if sexual cannibalism yields fitness benefits
for females, it is surprising that up to 35% of AM females do not cannibalize their mates
(given that they receive sperm despite cannibalism, Andrade 1996, 1998). Finally, if female
‘resistance’ behaviour arises because of fitness costs of IM mating due to the lost opportunity
for cannibalism, one would not expect similar behaviours for non-cannibalistic *Latrodectus*
species. However, I have replicated this experiment with *L. hesperus*, a species where
cannibalism is rare and males generally polyandrous, and found similar inter-sexual
behaviours and delays to IM matings relative to AM matings (Appendix 2). Thus based on
the data presented here, and my understanding of the evolutionary ecology and biology of *L.
hasselti*, it seems likely that immature mating is not detrimental to female fitness, but may be
neutral or beneficial.

There are potential benefits of IM mating for females, including reproducing without
waiting to attract a male after moulting, and the attendant elimination of the need to advertise
receptivity as an adult. Fertility assurance through IM mating may be important since
approximately 17% of *L. hasselti* females remain unmated in nature (Andrade & Kasumovic,
2005), a risk that could significantly affect selection on female behaviour (Kokko & Mappes,
2005). Moreover, unmated adult *L. hasselti* females suffer decreased longevity relative to
mated females (Stoltz et al., 2010), increasing the risk associated with delays to mating. If
females do not mate as immatures, then they produce sex pheromones as adults that attract
males (Kasumovick & Andrade, 2004; Stolz et al., 2007), and this requires expenditure of
time and resources on advertising rather than on producing and developing of eggs, and
incurs mortality risks. Pheromone production has recently been shown to be physiologically costly and an honest signal related to female condition in some species of black widows and other invertebrates (Baruffaldi & Andrade, 2015; Harari et al., 2011; Lelito & Brown 2006, 2008; Liske & Davis 1987). Female spider’s sex pheromones not only attract males but may also attract predators like parasitoid wasps (Foelix, 2011), or other spiders (Pearson & Rypstra, 2001; Pearson et al., 2001). My results suggest that IM females do not produce pheromones as adults (Fig. 4-4), similar to the pattern for adult females which stop producing sex pheromones immediately after mating (Jerhot et al., 2010; Stolz et al., 2007). Female cessation of pheromone production may reduce costs associated with continuous courtship by males, and with polyandry. In many species multiple matings expose females to a higher predation risk, transmission of sexual disease of substance associated to male sperm that could affect female fitness (Chapman, 2006; Wigby & Chapman, 2005). In L. hasselti, adult females that mate with two males sequentially produce fewer egg sacs with fewer spiderlings in each sac than females that mate only once (Stoltz, 2010). IM females could be behaving similarly to AM females once they have sperm stored to fertilize their eggs, both to reduce the likelihood of polyandry, and to avoid the cost of sex pheromone production. This is in contrast to results in Drosophila melanogaster, in which females that were coerced to mate then remated more frequently and were more attractive to subsequent males than were females mated consensually (Dukas & Jongsma, 2012b). This supports the idea that coerced females continued to produce sex pheromones. The difference in these results may suggest that any potential costs of advertising after an IM mating outweigh potential benefits of acquiring additional potential mates and the ability to ‘trade up’.
Another reason why females may not advertise as adults is that IM matings may be achieved only by high quality males in nature—thus eliminating the need to advertise for additional mates. Males that are able to locate and identify immature females in the short window of time when this type of mating is possible in nature may have superior sensory or locomotory traits; and the ability to successfully open the female’s genitalia without harm requires coordination between the sensory and motor organs; both of which could be indicators of male fitness (Anderson, 1994; Bisazza et al., 2001; Eberhard, 1996, 2009, 2004). Similarly, female ‘scraping’ behaviours may ensure only persistent, vigorous males are able to mate; similar to typical mechanisms to evaluate male quality under classic female choice (Anderson, 1994; Eberhard, 1996, 2004). In my studies, as in others, it is difficult to distinguish between female efforts to avoid unwanted copulations and selective resistance of copulatory attempts as a mechanism of female choice (Arnqvist, 1992; Eberhard, 1996, 2009, 2004; Thornhill, 1980). However, it is suggestive that, despite the extreme female-biased size dimorphism in this species, immature females were not aggressive towards males outside of attempts to remove them from the abdomen. In other *Latrodectus* species, females kill approaching males when they are not receptive (Johnson et al., 2011). Not only was sexual cannibalism absent from IM matings in this study, but IM males were frequently successful at copulating twice.

Thus, although mating with immature females involves behaviour and inter-sexual interactions that may superficially appear to be ‘coercive’, overall, this study suggests that this form of mating is neutral or beneficial to female fitness in *L. hasselti*. Moreover, even though I did not find short-term benefits of immature mating for females, they still may benefit overall if their male offspring are more likely to achieve ‘coercive’ matings.
themselves (e.g., West-Eberhard, 2014). These results also suggest the importance of simultaneous analysis of inter-sexual behavioural interactions and fitness correlates, and interpretation in terms of the natural history of particular species. Such studies may be critical to progress in understanding the taxonomic distribution of these types of tactics and thus rigorous tests of hypotheses for their evolutionary origin and maintenance.
References


Table 4-1

Description and comparison (mean ± s.d.) of male-initiated behaviours and mating outcomes scored for pairings between adult males and immature or adult females.

<table>
<thead>
<tr>
<th>Variable*</th>
<th>Description</th>
<th>Immature mated</th>
<th>Adult mated</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact latency (min)</td>
<td>Time from initiation of trial until the male first comes in contact with any part of the female’s body.</td>
<td>38 ± 59.94 (19)</td>
<td>26.89 ± 68.49 (19)</td>
<td>GLMS X² = 0.617, p = 0.432</td>
</tr>
<tr>
<td>Number of Contacts</td>
<td>Number of times the male touches the female prior to the first mounting attempt.</td>
<td>2.37 ± 3.33 (19)</td>
<td>1.05 ± 1.05 (19)</td>
<td>GLMS X² = 9.105, p = 0.003</td>
</tr>
<tr>
<td>Mount Latency (min)</td>
<td>Time from initiation of trial until the male first climbs onto the female’s abdomen</td>
<td>48.21 ± 60.63 (19)</td>
<td>39.78 ± 73.01 (19)</td>
<td>GLM X² = 0.252, p = 0.616</td>
</tr>
<tr>
<td>Number of Mounts 1</td>
<td>Number of times the male mounts the female prior to the first copulation.</td>
<td>7.63 ± 11.63 (19)</td>
<td>19.10 ± 8.55 (19)</td>
<td>GLMS X² = 87.254, p &lt; 0.001</td>
</tr>
<tr>
<td>Copulation 1 Latency</td>
<td>Time from start of trial to start of first copulation.</td>
<td>112.47 ± 92.96 (19)</td>
<td>145.68 ± 93.23 (19)</td>
<td>GLMS X² = 1.347, p = 0.246</td>
</tr>
<tr>
<td>Copulation 1 Duration (min)</td>
<td>Total duration of the first copulation (total time during which the first palp is inserted).</td>
<td>16.37 ± 6.3 (19)</td>
<td>16.16 ± 8.32 (19)</td>
<td>GLMS X² = 0.008, p = 0.930</td>
</tr>
<tr>
<td>Latency to Remount (min)</td>
<td>Time between the end of the first copulation and the male’s next mount of the female (excludes those males that did not dismount after the first copulation)</td>
<td>5.42 ± 12.20 (8)</td>
<td>13.92 ± 9.12 (13)</td>
<td>GLMS X² = 0.233, p = 0.629</td>
</tr>
<tr>
<td>Number of Mounts 2</td>
<td>Number of times the male mounts the female between the first and second copulation.</td>
<td>0.37 ± 0.50 (19)</td>
<td>3.08 ± 1.89 (13)</td>
<td>GLMS X² = 26.837, p &lt; 0.001</td>
</tr>
<tr>
<td>Copulation 2 Latency (min)</td>
<td>Time from end of 1st copulation to start of 2nd copulation.</td>
<td>18.5 ± 15.93 (18)</td>
<td>31.85 ± 20.78 (13)</td>
<td>GLMS X² = 3.703, p = 0.054</td>
</tr>
<tr>
<td>Copulation 2 Duration (min)</td>
<td>Total time during which the second palp is inserted.</td>
<td>24 ± 11.53 (18)</td>
<td>16.07 ± 7.52 (13)</td>
<td>GLM F = 4.673, p = 0.0039</td>
</tr>
<tr>
<td>Trial total duration (min)</td>
<td>Time from the initiation of the trial until the end of the final copulation achieved</td>
<td>185.58 ± 116.09 (19)</td>
<td>197.58 ± 112.24 (19)</td>
<td>GLMS X² = 0.130, p = 0.718</td>
</tr>
</tbody>
</table>

*Variables in bold differ significantly between the two treatments.
Table 4-2

Description and comparison (mean ± s.d.) of female sexual performance for pairings between adult males and immature or adult females.

<table>
<thead>
<tr>
<th>Variables*</th>
<th>Description</th>
<th>Immature mated</th>
<th>Adult mated</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Times female remove males when they try to approach</td>
<td>Females scrape legs against the body of a male that is mounted on the female’s abdomen or in copula</td>
<td>0.90 ± 1.48 (19)</td>
<td>0.26 ± 0.81 (19)</td>
<td>GLMS X2 = 5.786, p = 0.016</td>
</tr>
<tr>
<td><strong>Removal Attempts:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Before 1st copulation</td>
<td>3. ± 3.45 (19)</td>
<td>0.73 ± 1.24 (19)</td>
<td>GLMS X2 = 22.155, p &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>• During 1st copulation</td>
<td>0.79 ± 0.79 (19)</td>
<td>0.42 ± 0.51 (19)</td>
<td>GLMS X2 = 2.062, p = 0.151</td>
<td></td>
</tr>
<tr>
<td>• Between 1st and 2nd copulations</td>
<td>0.77 ± 1.31 (18)</td>
<td>0.14 ± 0.36 (14)</td>
<td>GLMS X2 = 5.025, p = 0.025</td>
<td></td>
</tr>
<tr>
<td>• During 2nd copulation</td>
<td>2.16 ± 3.27 (18)</td>
<td>0.77 ± 0.60 (13)</td>
<td>GLMS X2 = 8.535, p = 0.003</td>
<td></td>
</tr>
<tr>
<td>Total Movement (min)</td>
<td>Cumulative time spent moving across the web by the female during a mating trial.</td>
<td>22.32 ± 29.63 (19)</td>
<td>8.87 ± 7.07 (16)</td>
<td>GLMS X2 = 9.081, p = 0.003</td>
</tr>
</tbody>
</table>

*Variables in bold differ significantly between the two treatments.
Figure 4-1. The external genitalia of *L. hasselti* females, located on the ventral surface of the abdomen just anterior to the upper margin of the ‘hourglass’ marking, and epigastric furrow. In all images, the posterior is left, anterior is right. (A) Protuberant genital region of an immature female two days before molt to adulthood, no external openings are present. (B) Genital region of immature female after mating with an adult male with haemolymph visible leaking from the region that was opened by the male. (C) Epigynum (mature external genitalia) of an adult virgin female with genital openings uncovered following the final moult.

Figure 4-2. Relationship between the number of times immature or mature *L. hasselti* females scrape at males mounted on their abdomen as a function of the latency to copulation in laboratory trials.

Figure 4-3. Comparison (mean ± s.e.) of female fertility (100* [number of spiderlings in the first egg sac/total number of viable eggs], white bar), fecundity (total number of viable eggs, striped bar), latency to produce the first egg sac (number of days since adulthood day for IM females, and since mating day for AM females, grey bar) and longevity (number of days since adulthood day for IM females, and since mating day for AM females, black with white dots bar) for immature mated females (IM) and adult mated females (AM).

Figure 4-4. Comparison (mean ± s.e.) of male searching behaviour in 60 min trials on silk extracts from virgin females (V) (black bar), immature mated females (IM) (grey bar), adult mated females (AM) (striped bar) and control (methanol, C) (white bar). In the post-hoc test (Fisher’s Least significant difference, LSD) males searched and courted more on the V females silk extracts that the other females groups (V-IM: mean difference = 701.87, p = 0.025; V-AM: mean difference = 799.29, p = 0.009). However males spend roughly the same amount of time searching on silk extract from IM and AM (IM-AM mean difference = 97.42, p = 0.672).
Figure 4-1
Figure 4-3

Error Bars: +/- 1 SE
Figure 4-4
General Discussion

Understanding how sexual selection (Darwin, 1871) affects the evolution of sexual signals requires a thorough understanding of how species diverge and discriminate conspecific from heterospecifics (Andersson, 1994; Darwin, 1871), and also how isolated signals or signals combined with other information (cues) are used for mating decisions. Likewise, studies isolating the effects of communicatory channels and signals are required to identify traits under selection, and their role in the process of speciation (Andersson, 1994; Bradbury & Vehrencamp, 2011; Coyne & Orr, 2004). In my thesis I investigated factors affecting the diversity of signals at two levels--among species and within species--in a relatively sparsely studied signal modality (chemical signals), in a sparsely studied taxon (spiders). Most previous studies about the utilization and divergence of chemical signals have been performed in insects, particularly in pest insects, which limit our ability to infer broad patterns about mechanisms and effects of divergence in other taxa.

The widow spiders (family Theridiidae, genus *Latrodectus*) provide a unique opportunity to investigate sexual signal evolution, particularly of sex pheromones, because this taxon includes species with a large diversity of ecologies, different degrees of sympatry and allopatry with congeners, invasion history and mating behaviours (e.g., Andrade, 1996; Breene & Sweet, 1985; Garb et al., 2004; Forster, 1992; Kaston, 1970; Knoflach & van Harten, 2002; MacLeod, 2014; Ross & Smith, 1979; Segoli et al., 2006; Segev et al., 2003). These factors could shape evolutionary changes and plasticity in communication not only among individuals from the same species, but also among different species. Moreover, this genus is virtually unique among spiders because it combines three factors that are critical to
success of a comparative study of pheromone function: (1) there is a well-supported, species-level phylogeny (Garb et al., 2004; Condy et al., in prep), (2) sex pheromones have been chemically characterized in several species (Jerhot et al., 2010; Scott et al., 2015; Kiefer, Baruffaldi, Andrade, & Schulz et al., in progress), and extensive past studies of sexual selection have established well worked-out methodologies for assessing responses to pheromones and mating behaviour in the laboratory (e.g., Baruffaldi & Andrade, 2015; Perampaladas et al., 2008; Stoltz et al., 2007). These characteristics made this genus a key candidate, and potential model system, for testing hypothesis about the mechanism underlying patterns of pheromone diversity and divergence (Symonds & Elgar, 2008). Here I use focal species from different clades/biogeographical regions, with different mating systems to show that males of some species are able to use chemical cues alone (sex pheromones) to discriminate conspecific from heterospecific females (Chapter 2), food deprived potential cannibals from well fed females (Chapter 3), and immature-mated from adult-unmated females (Chapter 4).

Previous studies in insects showed that in some taxa, divergence in sex pheromones was positively correlated with genetic distance or divergence between the species, and this was particularly true if pheromones were not necessary to avoid hybridization (e.g., other cues or interactions served this function), or the species were geographically or ecologically isolated (Symonds & Elgar, 2008). This pattern is consistent with conclusions from the classic meta-analysis by Coyne & Orr (1989), in which *Drosophila* species pairs showed more pre- and post-zygotic isolation (pre-mating discrimination or failed hybridization) when they were more distantly related; but the degree of pre-zygotic isolation was higher in sympatric relative to allopatric species pairs (also see Coyne & Orr, 1997). Comparable
analyses of general hypotheses about links between evolutionary divergence and species isolation are rare (e.g., Funk et al., 2014; Tilley et al., 1990), likely due to the difficulty in acquiring data on behavioural isolation (inter-species matings), the outcome of hybridization, and genetic (or electrophoretic) data on divergence in a taxon with known evolutionary history (Coyne & Orr, 1997). To my knowledge, there are currently no comparative analyses of divergence in sex pheromones. Many of the studies that approach questions about speciation and sexual signals are lacking some or most of the necessary data, and so provide some, but incomplete information about divergence. In spiders for example, studies have shown that males of some species court and mate with heterospecific females (Costa-Smith & Machado, 2012; Kaston, 1970; Olive, 1982; Robert & Uetz, 2004 a, b; Schmidt, 1991; Stratton & Uetz, 1983), and in some cases are able to produce viable offspring (Costa & Capocasale, 1984; Schmidt, 1991; Stratton & Uetz, 1983). However, there is very little information about whether signals alone (particularly isolated sex pheromone -- without the silk as an additional source of cues) provide males with specific information about species identity (Gaskett, 2007; Schulz, 2004, 2013). Similarly, the few previous studies about inter-species discrimination in widows and in other spiders species did not take into account the phylogenetic relationship or genetic divergence between the species used (Cerveira & Jackson, 2013; Cross & Jackson, 2013; Roland, 1984; Nelson et al., 2012; Schulz & Toft, 1993; Trabalon et al., 1996; Willy & Jackson, 1993). Both types of information are critical to allow insights into whether the evolution of (chemical) communication between species follows the predicted pattern, as was observed in some insects (Symonds & Wertheim, 2005; Symonds & Elgar, 2008; Symonds et al., 2009).
In chapter two of my thesis I examine pheromone functional diversity using a chemical and behavioral perspective. Using focal widow species representing different clades and biogeographic regions I show that, consistent with a hypothesis of gradual evolution in allopatry, male *L. geometricus*, the most distantly related of my focal species, responded only to silk extracts from conspecifics, whereas *L. hesperus* responded only to the most closely related species. Overall, a comparison of genetic divergence compared to male responses to extracted pheromones of females across my 6 focal species were consistent with the predicted pattern (Fig. 2-2, 2-3). However, some of the details of inter-specific responses suggest other factors may shape discrimination. For example, *L. mirabilis* and *L. hesperus* did not exhibit a very clear pattern of discrimination with other species, although they had high mutual attraction. This may suggest the possibility of an adaptive convergent evolution in signals between these two species. Convergent or parallel evolution in the utilization of signals have been documented for acoustic, visual, vibrational and chemical signals in other taxa, in which environmental similarities (e.g., in transmission characteristics) or adaptation to comparable niches could affect the evolution and the utilization of some signals (Boughman, 2002; Harmon et al., 2005; Henry et al., 2012; Losos, 1992). *L. mirabilis* and *L. hasselti* could share some common components of their pheromone that have diverged in other species, and that could be maybe attributed to similar latitude or historical biogeography. This chapter is one of the few comparative tests of evolutionary divergence in the composition of or response to sex pheromones, and is also one of the most comprehensive analyses of arachnid chemical communication available to date. This work will be the backbone for later, more comprehensive comparative analyses of signal evolution and speciation in arachnids.
Sex pheromones may not only vary among species, but also between individuals from the same species (Johansson & Jones, 2007; Thomas, 2011). Among invertebrates, it has been shown that males can discriminate between chemical cues from mated and unmated females (Gaskett, 2007; Johansson & Jones, 2007; Thomas, 2011), and also from fed females and those that have had food withheld or reduced (Harari et al., 2011; Lelito & Brown, 2008; MacLeod & Andrade, 2014; Maxwell et al., 2010). Moreover, in some species it has been shown that mated females produce different chemicals, or different concentrations of chemicals than do unmated females (Ayasse et al., 1999; Baruffaldi et al., 2010; Carriere & McNeil, 1990; Stolz et al., 2007; Trabalon et al., 1996), and the same has been observed for females on different diets (Harari et al., 2011). In chapter three I examine whether females’ sex pheromones allow males to detect differences in female food intake and mass in two focal species of widow spiders (*Latrodectus hesperus* and *Latrodectus hasselti*), in which males experience different levels of risk of sexual cannibalism. I show that males of *L. hesperus* were less responsive to sex pheromones from food-deprived females compared to well-fed females whereas *L. hasselti* males showed similar responses to conspecific females in the same diet treatments. Females of both species lost more than half of their mass when food was withheld, and silk production was reduced, although these changes are not directly correlated with male sexual responses to pheromones (which are deposited by females in the silk). While females on good diets provide males with the benefit of higher fecundity in both species (Bonduriaiski, 2001; Edward & Chapman, 2011), the risk of being cannibalized by hungry females during courtship exists only in *L. hesperus*. Thus this species difference in male response suggests that male preferences in these spiders may depend less on the benefit
of seeking a highly fecund female and more on avoiding the cost of risky mating attempts with a likely cannibal. This hypothesis warrants future comparative study.

In Chapter 4, I focus on plasticity in pheromone production among females, and the insights this may provide regarding an unusual mating tactic of males in a focal species. In many animals, sexual selection on males to access females has driven the evolution of a wide range of behaviors (Parker, 1979), including a variety of vigorous sexual displays that may persuade females to mate (Andersson, 1994; Candolin, 2003), and also structural adaptations and coercive behaviours that force females to mate (Arnvist & Rowe, 2005; Chapman, 2006; McKinney et al., 1983). In this chapter, I assess effects of immature-mating on female mate attraction, mating tactics, and reproduction to examine fitness consequences for females. I show that *Latrodectus hasselti* females that mate as immatures have similar fecundity, fertility and longevity as adult-mated females even though the males rip the female’s exoskeleton and mate without courting. Monogyny, high mate-search mortality, and first-male sperm precedence may favor this tactic in males, but it was not known how it affected the female’s fitness. My work suggests that immature-mating may not be maladaptive for females. Consistent with this idea, relative to young virgin adults, immature-mated females show decreased pheromone production as do similarly-aged adult-mated females (Perampaladas et al., 2008; Stolz et al., 2007). Variation in female production of sex pheromones can limit male mating opportunities, and allow females control over the opportunity for polyandry (Andersson, 1994; Eberhard, 1996). Therefore, being mated as an immature could be beneficial for females which can avoid the risk of remaining unmated as adults, while minimizing energy expenditure for mate attraction.
**Future Directions**

My thesis has successfully identified how sex pheromones alone could be important mechanisms mediating species isolation in spiders, and for the first time in arachnids, I show that males from some species exhibit a phylogenetically-linked pattern of discrimination (Chapter 2). Moreover, I show that some cues reduce discrimination, even among species in which males clearly distinguish conspecifics based on signals (Chapter 2). Not only are sex pheromones important in some situations for species discrimination, but I also show that males can use the information encoded in female sex pheromones to assess feeding condition of conspecific females (Chapter 3) and for making mating decisions (Chapter 4). However, a number of questions remain unanswered at each level of communication (interspecific and intraspecific). Here, I outline what I consider to be some of the most important questions that would benefit from future consideration.

In chapter 2 I argue that widow spiders exhibit a phylogenetic pattern of discrimination using sexual signals, particularly sex pheromones, supporting the previous observation in wolf spiders (Robert & Uetz, 2004a). However, many questions remain about the divergence and diversity of the chemical composition of the sex pheromones in widow spiders and in spiders in general. Currently, there are two pheromones identified in this genus (from a total of eight known across all spiders, Schulz, 2013); the pheromones of *L. hasselti* and *L. hesperus*, which seems to be isomers of each other (Jerhot et al., 2010; Scott et al., 2015). During my thesis research, together with collaborators, I also identified the sex pheromone of *L. geometricus* (Appendix 1, Kiefer, Baruffaldi, Andrade & Schulz in progress). This pheromone could provide key information about the diversification of sex.
pheromones in *Latrodectus* because it is a species from a different clade and the most discriminating of all species tested here. However, more target species need to be added in order to get a better understanding of the evolution of sex pheromones in spiders (Symonds & Wertheim, 2005; Symonds & Elgar, 2008; Symonds et al., 2009). Even though individual chemical components may be sufficient to elicit male responses, it is important to consider the possibility that *Latrodectus* sex pheromones are a ‘cocktail’ of chemicals, some of which may be common across species, and/or reduce male activity. In particular, the chemical identification of the sex pheromone in *L. mirabilis* could bring important insights given my proposal of convergent evolution in pheromone composition with *L. hasselti* (Chapter 2). In addition, in my thesis I only used allopatric species, thus in the future a focus on sympatric species will provide valuable information about the effect of the hybridization risk on the evolution of sex pheromones (Symonds & Elgar, 2008).

In chapter three I showed that in some species where the risk of pre-copulatory sexual cannibalism is high, males used information encoded in the females’ pheromones to avoid hungry, potentially cannibalistic females. However, when this risk is lower, and/or the chance of future matings with other females is sufficiently low (e.g., high rates of post-copulatory sexual cannibalism), males are less likely to pay attention to that information. Future research could investigate if the composition or concentration of the sex pheromones produced by hungry and well-fed females differs. If females produce different pheromones (quality or quantity) according to their feeding condition, as I proposed, this could provide valuable information about males’ ability to discriminate between these groups of females, independent of their chosen sexual response once the information is received. Although I argued that males could discriminate among females in both focal species, I was unable to
test whether *L. hasselti* males were similarly attracted to fed and unfed females because information of female feeding history was absent, or whether these males were simply less likely to pay attention to that information. In some species of insects has been shown that females with different diets produce different blends of chemicals and that males use that information to evaluate future matings (Harari et al., 2011). Together with a mechanistic analysis of female pheromone production, these results suggest a comparative test of the cannibalism hypothesis for the evolution of male preferences for well-fed females is warranted.

In chapter four I propose that immature mating could be beneficial for females, because immature-mated females had similar fecundity, fertility, longevity and sex pheromone production as adult-mated females. Previous observations in the field suggested that immature females that were in proximity to adult virgin females had a higher chance of being mated as immatures, than those that were isolated or surrounded by other immature females (MCB unpublished data). Those observations, in addition to the findings of this thesis, lead to new questions about the mechanisms males use to localize immature females in nature. It has been shown in other spider species that males cohabit with immature females until adulthood, likely to secure virgin females, and that immature females could be producing pheromones when they are close to adulthood (Gaskett, 2007; Huber, 2005; Herberstein, 2011; Jackson, 1986a,b). Immature females of *L. hasselti* provide an excellent study subject to test new hypothesis about male attraction by juveniles, and/or the ability of males to locate cryptic immature females when they are physiologically able to mate. Understanding mate attraction and mate localization could provide important insights into the selective pressures acting on each sex, and how these may vary with the operational sex ratio.
Immature-mating could decrease the intensity of sexual selection on otherwise monogynous males in these widow species, and females that may face the risk of remain unmated (Huber, 2005). However, monogyny does not appear to be necessary for immature mating to evolve, as shown by my own additional study of immature mating in the polyandrous (MacLeod, 2014) species, *L. hesperus* (Appendix 2). From a broader perspective, the fact that this behaviour is not necessarily coupled to the risk of sexual cannibalism in *Latrodectus* suggests that immature-mating may be a widespread tactic in other invertebrates, particularly when unmated females are of high reproductive value, when males associate with immature females in nature, and if males possess structures (e.g. fangs in spiders) that can be used to access developing genitalia of females. Therefore, more studies looking in detail at male mate-guarding of immature females could provide more information about whether this behavior is widespread in nature.

**Conclusion**

Diversification is sexual signals arises principally due to genetic drift, sexual selection acting in reproductive isolation, and/or natural selection acting on ecological adaptation or sensory drive (Anderson, 1994; Boughman, 2002; Coyne & Orr, 2004; Harmon et al., 2005; Henry et al., 2012; Huber, 2005; Kirkpatrick & Ryan, 1991; Losos, 1992; Philips & Johnston, 2009; Simmons & Elgar, 2008). Prior studies of sex pheromone evolution and diversification in pest insects identified and reported two patterns of diversification among species (phylogenetic distance and reproductive isolation), but few studies have been comprehensive combinations of chemical information with behavioural information. More accurate information about signal diversification and inferences about likely effects in nature could be
obtained by studies that incorporate simultaneously the impact of male discrimination of cues alone in conjunction with effects of male-female interaction (Coyne & Orr, 1997; Symonds & Elgar, 2008). Studies that isolate one communicatory channel are fundamental as they can determine whether a trait is selected, but conclusions about global effects may be more informative when pre and post zygotic isolation among species is studied more broadly. My thesis has investigated the influence of chemical cues alone, and male behaviour in the presence of females, providing valuable information about the importance of the different levels of communication and discrimination between and within species.
References


Appendix 1

The methanol extractable pheromone from females of *L. geometricus* was identified by GC/MS and NMR analysis of extracts of silk from unmated adult females, juveniles, and males. Synthesis and stereochemical analysis revealed the pheromone to be N-3-methylbutyryl-O-propionyl-L-serine methyl ester (Fig. A-1-1). Male mate searching behaviour was analyzed when males were exposed to the synthetic candidate pheromone, the other stereoisomer (N-3-methylbutyryl-O-propionyl-D-serine methyl ester), and a control (methanol) using the same methodology as in Jerhot et al., 2010. I found that only the L-enantiomer produced a significant sexual response in males (GLM (gamma distribution): likelihood ratio: $\chi^2_1 = 14.198, p < 0.001$, Fig. A-1-2).
Figure A-1-1. Proposed stereochemical from the pheromone of *L. geometricus* virgin females: N-3-methylbuttyryl-O-propionyl-L-serine methyl ester.

Figure A-1-2. Comparison (mean ± s.e.) of male searching behaviour in 60 min trials on extracts from the L-enantiomer (n = 18, black bars) and D-enantiomer (n = 11, grey bars, [0.5mg synthetic pheromone/1ml of methanol]) from the preliminary sex pheromone candidate from virgin females of *L. geometricus*. Mean search values are raw search times for each treatment minus the average male activity on methanol controls (n = 18, n = 11) in comparable trials.
Figure A-1-1
Figure A-1-2
Appendix 2

To test if immature mating was necessarily associated with monogyny, I studied the occurrence of immature mating in species *L. hesperus*, a congener of *L. hasselti* in which there is no post-sexual cannibalism and males are able to re-mate up to 4 times under laboratory conditions (MacLeod, 2014). I used the same methodology outlined in Chapter 4.

I found that *L. hesperus* males also mate with immature females and do not perform courtship behavior, like occurs in *L. hasselti*, and also that males achieve the same frequency of mating success with immature (42% success) and adult (42% success) females in laboratory conditions.

Moreover, as with *L. hasselti*, immature-mated females had similar fertility (AM n = 9, IM n =12, ANOVA: F = 0.1185, p = 0.7344), fecundity (AM n = 9, IM n =12, Mann-Whitney: U = 44, z =-0.6773, p = 0.4982), and longevity (AM n = 9, IM n =11, ANOVA: F = 1.121, p = 0.3037) as adult mated females (Fig. A-2-1).

Furthermore, male responses to the extract from silk of immature-mated females suggest that they do not produce sex pheromones after mating (and moulting), like adult mated females (Fig. A-2-2). These results are in line with I showed for *L. hasselti* females in chapter 4.
Figure A-2-1. Comparison (mean ± s.e.) of *Latrodectus hesperus* female fertility (number of spiderlings in the first egg sac), fecundity (fertility/number of viable eggs), and longevity (number of days since adulthood day for IM females, and since mating day for AM females) for immature mated females (IM, gray) and adult mated females (AM, black).

Figure A-2-2. Comparison (mean ± s.e.) of *Latrodectus hesperus* male searching behaviour in 60 min trials on silk extracts from virgin females (V) (striped bar), immature mated females (IM) (grey bar), adult mated females (AM) (black bar) and control (methanol, C) (white bar). Males spent much time searching and courting on the silk extract of virgin females than the time spent moving on the solvent-only control (n = 15, GLMS: Wald-$\chi^2 = 4.041$, p = 0.044). Male response to the control was similar to responses when males were exposed to extracts of silk of AM females (n = 8, GLMS: Wald-$\chi^2 = 1.057$, p = 0.304) or of IM females (n = 9, GLMS: Wald-$\chi^2 = 0.050$ p = 0.822), suggesting a similar lack of pheromone activity.
Figure A-2-1

![Graph showing mean values for Fecundity, Fertility, and Longevity across different treatments. Error bars indicate ±1 SE.](image)
Figure A-2-2

[Bar graph showing mean values (min) for different treatments: Methanol=Control, AM, IM, V. Error bars indicate ±1 SE.]
Factors affecting male activity on pheromone extracts (target variable) were examined using generalized linear mixed models for each of two *Latrodectus* species. Corrected Akaike Information Criterion (AICc) values were used to determine the best model. Initial models included all putative fixed factors, which were dropped (**) from the model in descending order according to *P* value.

<table>
<thead>
<tr>
<th>Species</th>
<th>Fixed factors</th>
<th>AICc</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. hesperus</em></td>
<td><strong>Model 1</strong></td>
<td>239.281</td>
<td>$F_{5,87} = 2.340; p = 0.048$</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td>$F_{1,87} = 2.091; P = 0.152$</td>
</tr>
<tr>
<td>Female mass</td>
<td></td>
<td></td>
<td>$F = 0.471; p = 0.494$</td>
</tr>
<tr>
<td>Silk mass</td>
<td></td>
<td></td>
<td>$F = 0.002; p = 0.962**$</td>
</tr>
<tr>
<td>Trt*fem mass</td>
<td></td>
<td></td>
<td>$F = 0.247; p = 0.621$</td>
</tr>
<tr>
<td>Trt* silk mass</td>
<td></td>
<td></td>
<td>$F = 2.174; p = 0.144$</td>
</tr>
<tr>
<td><strong>Model 2</strong></td>
<td></td>
<td>239.281</td>
<td>$F_{5,87} = 2.340; p = 0.048$</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td>$F_{1,87} = 2.091; p = 0.152$</td>
</tr>
<tr>
<td>Female mass</td>
<td></td>
<td></td>
<td>$F_{1,87} = 0.471; p = 0.494$</td>
</tr>
<tr>
<td>Trt*fem mass</td>
<td></td>
<td></td>
<td>$F_{1,87} = 0.247; p = 0.621**$</td>
</tr>
<tr>
<td>Trt* silk mass</td>
<td></td>
<td></td>
<td>$F_{2,87} = 2.075; p = 0.132$</td>
</tr>
<tr>
<td><strong>Model 3</strong></td>
<td></td>
<td>230.534</td>
<td>$F_{4,88} = 2.890; P = 0.027$</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td>$F_{1,88} = 3.045; p = 0.084$</td>
</tr>
<tr>
<td>Female mass</td>
<td></td>
<td></td>
<td>$F_{1,88} = 0.212; p = 0.647**$</td>
</tr>
<tr>
<td>Trt*silk mass</td>
<td></td>
<td></td>
<td>$F_{2,88} = 2.722; p = 0.071$</td>
</tr>
<tr>
<td><strong>Model 4</strong></td>
<td></td>
<td>218.649</td>
<td>$F_{3,89} = 3.795; p = 0.013$</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td>$F_{1,89} = 11.087; p = 0.001$</td>
</tr>
<tr>
<td>Model</td>
<td>$F$-value</td>
<td>$p$-value</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>Model 5</td>
<td>252.587</td>
<td>$F_{2,89} = 2.980; p = 0.056^{**}$</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td>$F_{1,91} = 4.607; p = 0.035$</td>
<td></td>
</tr>
<tr>
<td><strong>L. hasselti</strong> Model 1</td>
<td>262.102</td>
<td>$F_{5,86} = 0.550; p = 0.738$</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td>$F_{1,86} = 0.273; p = 0.603$</td>
<td></td>
</tr>
<tr>
<td>Female mass</td>
<td></td>
<td>$F_{1,86} = 1.687; p = 0.197$</td>
<td></td>
</tr>
<tr>
<td>Silk mass</td>
<td></td>
<td>$F_{1,86} = 0.012; p = 0.911^{**}$</td>
<td></td>
</tr>
<tr>
<td>Trt*fem mass</td>
<td></td>
<td>$F_{1,86} = 0.315; p = 0.576$</td>
<td></td>
</tr>
<tr>
<td>Trt*silk mass</td>
<td></td>
<td>$F_{1,86} = 0.542; p = 0.464$</td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td>262.102</td>
<td>$F_{5,86} = 0.550; p = 0.738$</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td>$F_{1,86} = 0.273; p = 0.603$</td>
<td></td>
</tr>
<tr>
<td>Female mass</td>
<td></td>
<td>$F_{1,86} = 1.687; p = 0.197$</td>
<td></td>
</tr>
<tr>
<td>Trt*fem mass</td>
<td></td>
<td>$F_{1,86} = 0.315; p = 0.576$</td>
<td></td>
</tr>
<tr>
<td>Trt*silk mass</td>
<td></td>
<td>$F_{2,86} = 0.496; p = 0.611^{**}$</td>
<td></td>
</tr>
<tr>
<td>Model 3</td>
<td>293.750</td>
<td>$F_{4,88} = 0.413; p = 0.744$</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td>$F_{1,88} = 0.293; p = 0.590^{**}$</td>
<td></td>
</tr>
<tr>
<td>Female mass</td>
<td></td>
<td>$F_{1,88} = 1.111; p = 0.295$</td>
<td></td>
</tr>
<tr>
<td>Trt*silk mass</td>
<td></td>
<td>$F_{2,88} = 0.895; p = 0.347$</td>
<td></td>
</tr>
<tr>
<td>Model 4</td>
<td>296.533</td>
<td>$F_{2,89} = 0.602; p = 0.550$</td>
<td></td>
</tr>
<tr>
<td>Female mass</td>
<td></td>
<td>$F_{1,89} = 1.103; p = 0.296^{**}$</td>
<td></td>
</tr>
<tr>
<td>Female mass * trt</td>
<td></td>
<td>$F_{1,89} = 1.155; p = 0.285$</td>
<td></td>
</tr>
<tr>
<td>Model 5</td>
<td>296.533</td>
<td>$F_{2,89} = 0.602; p = 0.550$</td>
<td></td>
</tr>
<tr>
<td>Female mass * trt</td>
<td></td>
<td>$F_{2,89} = 0.602; p = 0.550$</td>
<td></td>
</tr>
</tbody>
</table>