Chemical communication of the false widow spiders *Steatoda grossa* and *S. triangulosa* (Theridiidae)

by

Andreas Fischer

M.P.M., Simon Fraser University, 2023
M.Sc. (Dist.), Ulm University, 2016

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy

in the
Department of Biological Sciences
Faculty of Science

© Andreas Fischer 2023
SIMON FRASER UNIVERSITY
Summer 2023

Copyright in this work rests with the author. Please ensure that any reproduction or re-use is done in accordance with the relevant national copyright legislation.

Declaration of Committee

Name: **Andreas Fischer**

Degree: **Doctor of Philosophy**

Title: **Chemical communication of the false widow spiders *Steatoda grossa* and *S. triangulosa* (Theridiidae)**

Committee:

Chair: Norbert Haunerland
Professor, Biological Sciences

Gerhard Gries
Supervisor
Professor, Biological Sciences

Jennifer Cory
Committee Member
Professor, Biological Sciences

Leithen M'Gonigle
Examiner
Associate Professor, Biological Sciences

Jutta Schneider
External Examiner
Professor, Biology
University of Hamburg

Abstract

The chemical ecology of web-building spiders is poorly understood. Only 12 sex pheromones are known, the side of pheromone production remains elusive, a pheromonal function in female-female conflict has not been described, the effect of male pheromone on male copulatory success has rarely been studied, and there is no report whether female spiders recognize ‘self’. Addressing these knowledge gaps, I worked with the false black widow *Steatoda grossa,* and the triangulate cobweb spider, *S. triangulosa.*

I found that subadult female *S. grossa* stay cryptic to mate-seeking adult males, which is likely adaptive to sub-adult females that are in sexual conflict with adult males cohabiting their webs.

Working with adult female *S. grossa*, I identified three new contact pheromone components: *N*-4-methylvaleroyl-*O*-butyroyl-L-serine, *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine and *N*-4-methylvaleroyl-*O*-hexanoyl-L-serine. These compounds originate from the posterior aggregate silk gland, induce courtship by males, and web pH-dependently hydrolyse at the carboxylic-ester bond, giving rise to three corresponding carboxylic acids that attract males. A carboxyl ester hydrolase present on webs likely mediates the functional transition of contact sex pheromone components to the carboxylic acid mate attractant pheromone components.

Non-targeted metabolomics helped reveal the contact pheromone components of *S. triangulosa*: *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine, *N*-3-methylbutanoyl-*O*-isobutyroyl-L-serine, and *N*-3-methylbutanoyl-*O*-butyroyl-L-serine. Hydrolyses of these serine esters gives rise to butyric and isobutyric acids as mate-attractant pheromone components.

Female *S. grossa* sense intra-sexual competition via their sex pheromones, and respond to sexual, social and natural selection pressures originating from intra-sexual conflict. In settings of high intra-sexual competition, females adjusted their webs to increase prey capture and lower predation risk. To alleviate mate competition, females deposited more contact pheromone components on their webs and accelerated their breakdown to mate-attractant pheromone components, essentially increasing their webs’ attractiveness.

Web reduction behaviour by courting *S. grossa* males has no long-range effect on mate-

seeking males but functions as an inter-sexual signal. Courting males with functional (silk-releasing) spinnerets were more likely to copulate with the female than males with their spinnerets experimentally occluded. The male's signal likely entails a volatile silk-borne pheromone.

Female *S. grossa* indiscriminately accepted both their own webs and egg sacs and those of conspecific females, likely due to a lack of selection pressure to recognize 'self'.

Keywords: Sexual conflict; spider sex pheromone; same-sex conflict; multi-modal courtship; chemical signature

Dedication

I dedicate this Doctor of Philosophy thesis to my children Raphael and Lily. This degree is the outcome of persistence, perseverance when I wanted to quit, and the support I received from your mother, the Gries laboratory and simply obedience to God. Many times I wanted a very different route for my life, but the most satisfying place I learned to be is in the will of God for your life, for me it is Science, for you it might be something different, but when you are close to His heart you will never be off track. I have been blessed with the right people at the right time, with great ideas that lead to key discoveries of this thesis, but also with many difficulties through which I grew in patience. I wish you a fulfilled life that is not always easy but rewarding. Beyond any academic achievement I am grateful and proud to be your father.

Acknowledgements

I thank Prof. Dr. Norbert Haunerland for agreeing to chair my defence, as well as Prof. Dr. Leithen M'Gonigle to serve as public examiner and Prof. Dr. Jutta Schneider to serve as the external examiner. I also thank Dr. Mike Hart (DGSC Chair) and Laurie Sutterlin who made it possible to complete this PhD concurrently with my MPM.

Dr. Jenny Cory – I am incredibly grateful to you for years of invaluable guidance on the supervisory committee. Your expertise helped me craft the direction of my research.

Foremost, I thank my Doktoreltern senior supervisor, Prof. Dr. Gerhard Gries, and the unofficial senior supervisor, Regine Gries. You are not just supervisors, but mentors who went above and beyond to ensure my success. Your guiding mentorship has set me up to be a successful researcher because of your rigorous love for science and excellent research. You were the prime cheerleaders during my time at SFU, encouraging me and helping me steer clear of dead-ends. You have shaped my skills and character by modelling the life of scientists as a synergistic team. Thank you for the practical support by always providing the materials, equipment, analytical skills as well as financial support. I am also truly thankful for having given me the opportunity to present our research at many international conferences for which you paid, places like India, New Zealand, USA and countless Canadian cities would not have been visited without your generous support. Further, I appreciate your sense of humour, passion for science, and open-mindedness to new approaches that fostered the highly productive but also caring atmosphere in your lab. The 'there-will-be-sweets' meetings created a consistent platform for exchange of ideas, expertise, and excellence within the lab. Your open-door policy made you always available to address any issues that came up and I always left with a sense for the next steps. Your integrity, fairness and wisdom has left a mark on me. The success of this thesis rests exclusively on your mentorship.

One great joy of my PhD research was mentoring undergraduate students and collaborating with them in multiple research projects. I would like to thank Emmanuel

Hung for our lengthy discussions and jokes. You conducted excellent research as an undergraduate with me, and now I am impressed by your research program as my peer in the Gries lab. Further, I'd like to thank Rina (Yerin) Lee! Never have I met a more dedicated, motivated, and brilliant student than you. I am grateful for the friendship that grew out of our scientific collaborations, and I am proud that you are now in the medical school program of the UofT. I would like to thank Sula (Yasasi) Fernando for having been the most joyful and persisting student – you still hold the record of most consecutive runs at the HPLC. Your wit and brilliance have been dearly missed and with much pride I know you are thriving in your PhD program at the UofT. Next, I would like to thank Camilla (Andrea) Roman-Torres who despite the covid lockdowns pushed our scientific projects further while becoming a dear friend! I will never forget the weekly door-step deliveries of hundreds of false widows to remain productive. With a full heart I am grateful for the many different projects I was able to work with you. Dino (Xiang Hao) Goh, who is now a veterinarian in Australia, managed to start and finish his doctorate before I wrote this – thank you, brother, for the heart connection and all the best wishes conquering the world. Next, I'd like to thank the undergraduates whose directed study projects earned them a co-authorship to chapters of this thesis: Thank you Jordan Stewart, April Preston, Sarah Moniz-de-Sa, Jamie-Lynne S. Varney, Neilofar Amiri, Nastaran Bahar and Jane Vurdela.

I would like to thank Hongwen Chen for operating the LC-MS/MS and running the never-ending stream of thousands of samples over the various projects. Thank you, Adam Blake, Hanna Watkins, and Em Lim for advice on statistical tests and code debugging. I would like to thank Derrick Horne for assistance with scanning electron microscopy and Rainer Welzenberger for his useful advice on the stridulatory sound of European *Steatoda grossa*.

To my fellow Gries-lab members I am also grateful for their continuous support over the years. Special thanks to Dr. Steve Takács for your humorous support and the beautiful scientific artwork for my publications, to Dr. Santosh Alamsetti and Dr. Anand Devireddy for crafting the needed semiochemicals. I thank those Gries-lab members who have finished since I began: Josh Pol, Warren Wong, Tamara Babcock, Mike Hrabar, Dr.

Daniel Peach, Danielle Hoefele, Yonathan Uriel, Dr. Adam Blake, Elton Ko, Jaime Chalissery, Dr. Elana Varner, Emily Lemke, and Kendal Singleton. As well as my current lab members: Drs.to-be Asim Renyard, Sam Meraj and Emmanuel Hung, as well as the MPM candidates Saif Nayani, Claire Gooding, Emma Kovacs, Mikhaela Ong, and Thet Thet Zaw. The reader must acknowledge how exceptionally prolific Gerhard and Regine Gries operate their lab.

I am indebted to the Natural Sciences and Engineering Research Council of Canada for supporting me financially with the Alexander Graham Bell Scholarship. I would like to also thank Simon Fraser University for awarding me multiple Graduate Fellowships, the Dr. H. R. MacCarthy Graduate Bursary, and the President's PhD Scholarship. Lastly, I would like to thank Dr. Gerhard Gries for supporting me financially with multiple Research Assistant assignments. All your financial support made the scientific explorations of this thesis possible.

Lastly, I would like to thank my loving and supporting wife Alexandra. The many years of studying, researching, and writing have been a joy because of your encouragement. You supported my dreams and professional ambitions, even at the cost of wealth and long-term financial security. You supported me when things were tough and celebrated with me the many victories and breakthroughs. Your advice has been imperative and timely when it came to important decisions. But most of all I thank you for our awesome children, Raphael and Lily who bring so much joy, laughter, and bliss into my life. Thank you for being my friend, counselor, and partner. I love you.

Table of Contents

Declaration of Committee ii

Abstract iii

Dedication v

Acknowledgements vi

Table of Contents ix

List of Figures xii

List of Acronyms xix

Glossary xx

Chapter 1: Introduction 1

- 1.1 Chemical crypsis of subadult females 3
- 1.2 Origin and identification of contact and volatile sex pheromone components 4
- 1.3 Non-targeted metabolomics to aid pheromone identification. 5
- 1.4 Recognition of female-female conflict by mate-attractant pheromone components 6
- 1.5 Pheromones of spider males 8
- 1.6 'Mine or thine' chemical cues mediate recognition of self and non-self 9
- 1.7 Model spiders 9
- 1.8 Overview of research chapters 10
- 1.9 References 13

Chapter 2: Dodging sexual conflict? – Sub-adult females of a web-building spider stay cryptic to mate-seeking adult males[1] 20

- 2.1 Abstract 20
- 2.2 Introduction 21
- 2.3 Methods 23
- 2.4 Results 26
- 2.5 Discussion 27
- 2.6 Acknowledgments 29
- 2.7 References 29

Chapter 3: Origin, structure and functional transition of sex pheromone components in a false widow spider[1] 34

- 3.1 Abstract 34
- 3.2 Introduction 35

3.3 Results and Discussion 37
3.4 Conclusions 44
3.5 Methods 45
3.6 Acknowledgments 55
3.7 References 55
Chapter 4: Non-targeted metabolomics aids in sex pheromone identification – a proof-of-concept study with the triangulate cobweb spider, *Steatoda triangulosa*.[1] 68
4.1 Abstract 68
4.2 Introduction 69
4.3 Methods 71
4.4 Results 76
4.5 Discussion 77
4.6 Acknowledgements 80
4.7 References 81
Chapter 5: Same-sex conflict in a spider – Female false black widows adjust their webs' architecture and attractiveness in response to competition for prey and mates, and to predation risk.[1] 92
5.1 Abstract: 92
Keywords 92
5.2 Introduction 93
5.3 Materials and Methods 96
5.4 Results 101
5.5 Discussion 103
5.6 Acknowledgements 107
5.7 References 107
Chapter 6 Multimodal and multifunctional signaling? – Web reduction courtship behavior in a North American population of the false black widow spider[1] 117
6.1 Abstract 117
6.2 Introduction 118
6.3 Methods 120
6.4 Results 125
6.5 Discussion 127
6.6. Conclusion 130

6.7 Acknowledgments 131
6.8 References 131
Chapter 7: 'Mine or Thine' – Indiscriminate responses to own and conspecific webs and egg sacs by the false black widow spider, *Steatoda grossa* (Araneae:Theridiidae) [1] 141
7.1 Abstract 141
7.2 Introduction 142
7.3 Materials and methods 143
7.4 Results 145
7.5 Discussion 146
7.6 Acknowledgments 147
7.7 References 148
Appendix. Supplementary Information of Chapter 3 152

List of Figures

Figure 2.1 **Bioassay results**. Mean (+ SE) proportion of time that male *Steatoda grossa* (*i*) stayed on a test stimulus relative to the total bioassay time (green bars), and (*ii*) displayed courtship on a test stimulus relative to the time spent on it (red bars). In each of experiments 1-8 (N = 25 each), an asterisk (*) indicates a statistically significant behavioural response to a test stimulus (Mann-Whitney U test, $p < 0.05$). Note: (*i*) 1 WEE = 1 Web Extract Equivalent; (*ii*) lack of a red bar indicates no occurrence of courtship behaviour; (*iii*) males were not re-tested within experiments.

Figure 3.1: **Known contact pheromone components of spiders and methods to identify analogous components produced by *Steatoda grossa* females.** (**a**) Pheromone components of the spiders (*i*) *Linyphia triangularis* ([(*R*)-3-hydroxybutyryloxy-butyric acid (**1**) with its breakdown product (*R*)-3-hydroxybutyric acid (**4**)], (*ii*) *Latrodectus hasselti* ([*N*-3-methyl-butyryl-*O*-(*S*)-2-methylbutyryl-*L*-serine methyl ester (**2**)], and (*iii*) *Latrodectus hesperus* [(*N*-3-methylbutanoyl-*O*-methylpropanoyl-*L*-serine methyl ester (**3**)]. (**b**) Triangular prism scaffold for a female spider to build her web. (**c**) T-rod apparatus for testing courtship behaviour by *S. grossa* males in response to test stimuli (web extract or fractions thereof; synthetic candidate pheromone components; solvent control) applied to a piece of filter paper attached to each distal end of the horizontal arm. (**d**) Total ion chromatogram of compounds unique to sexually mature *S. grossa* females (pyrrolidin-2-one (**5**), 4-hydroxyhydrofuran-2(3*H*)-one (**6**), nonanoic acid (**7**), dodecanoic acid (**8**), 6-methylheptanamide (**9**), octanamide (**10**), 4,6-dimethyl heptanamide (**11**); identified by gas chromatography-mass spectrometry of crude female web extract. (**e**) Extent of courtship by *S. grossa* males in response to female web extract or synthetic candidate pheromone components. Circles and boxplots show the time single male spiders courted in each replicate and the distribution of data (minimum, first quartile, median, third quartile, maximum), respectively. Medians with different letters indicate statistically significant differences in courtship responses. Wilcoxon test, $P < 0.05$.

Figure 3.2: **Contact pheromone components of female *Steatoda grossa*.** (**a**) High performance liquid chromatogram (HPLC) of compounds [*N*-4-methylvaleroyl-*O*-isobutyroyl-*L*-serine (**12**); *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**16**); *N*-4-methylvaleroyl-*O*-hexanoyl-*L*-serine (**17**)] present in crude web extract of female *S. grossa,* and HPLC mass spectrum of **12** (with **16** coeluting). (**b, c**) Total ion chromatogram (**b**) and mass spectra (**c**) of compounds [*N*-4-methylvaleroyl-*O*-butyroyl-*L*-serine methyl ester (**13**), *N*-4-methylvaleroyl-*O*-isobutyroyl-*L*-serine methyl ester (**14**), *N*-4-methylvaleroyl-*O*-hexanoyl-*L*-serine methyl ester (**15**)]

identified by gas chromatography-mass spectrometry (GC-MS) in esterified web extract of female *S. grossa*. (**d**) Extent of courtship by male *S. grossa* in response to stimuli tested in T-rod bioassays. The names of compounds 5–11 are reported in the caption of Fig. 3.1. Circles and boxplots show the time single male spiders courted in each replicate and the distribution of data (minimum, first quartile, median, third quartile, maximum), respectively. Medians with different letters indicate statistically significant differences in courtship responses; Kruskal-Wallis χ^2 test with Benjamini-Hochberg correction to account for multiple comparisons, $P < 0.05$.

Figure 3.3: **Origin of contact pheromone components produced by female *Steatoda grossa***. (**a**) High performance liquid chromatography - mass spectrometry (HPLC-MS) quantification of two contact pheromone components [*N*-4-methylvaleroyl-*O*-isobutyroyl-*L*-serine (**12**) coeluting with *N*-4-methylvaleroyl-*O*-isobutyroyl-*L*-serine (**16**)], present in the abdomen and cephalothorax of female spiders. (**b**) HPLC-MS quantification of **12** & **16** in the hemolymph and various tissues of the abdomen. (**c**) HPLC-MS quantification of **12** & **16** in various silk glands. In each of experiments 19–21, circles and boxplots show the amount of **12** & **16** present in each spider and the distribution of data (minimum, first quartile, median, third quartile, maximum), respectively. Medians with different letters indicate significantly different amounts of **12** & **16** present in various sources; Wilcoxon and Kruskal-Wallis χ^2 test with Benjamini-Hochberg correction to account for multiple comparisons, $P < 0.05$.

Figure 3.4: **Transition of contact pheromone components produced by female *Steatoda grossa* to sex attractant pheromone components**. (**a**) Moving-air dual-choice Y-tube olfactometer. (**b**) Attraction of *S. grossa* males in Y-tube olfactometers to extracts of female webs and to volatile compound **5**–**11** (names in Fig. 3.1 caption) unique to sexually mature females. (**c**) Predicted breakdown of contact pheromone components **12**, **16**, and **17** to the amide **18** and the volatile carboxylic acid mate attractant pheromone components **19**, **20**, and **21**. (**d**) Breakdown rate of contact pheromone components [ratio of **18** / (**12** + **16** + **17** + **18**)] on webs extracted 0 or 14 days after being built; circles and boxplots show the breakdown rates of single webs and the distribution of data (minimum, first quartile, median, third quartile, maximum), respectively, at days 0 and 14, which differed significantly (Wilcoxon test, $P < 0.05$). (**e**) Attraction of *S. grossa* males in Y-tube olfactometers to single- or multiple-component blends of synthetic compounds; in each experiment, an asterisk denotes a significant preference for the treatment stimulus (one-tailed binomial tests; $P < 0.05$). (**f**) Captures of *S. grossa* males in 10 pairs of sticky traps that were deployed in building hallways between September and December 2018. During weekly checks, the position of the treatment and control trap within each pair was randomized; the treatment trap was baited with the

carboxylic acids **19**, **20**, and **21** (see Methods for detail), whereas the control trap was left unbaited; the asterisk denotes a significant preference for the treatment trap (one-tailed binomial test; $P < 0.05$).

Figure 3.5: **pH-dependent breakdown of contact pheromone components**. (**a**) Relationship between the pH of female *Steatoda grossa* webs and the breakdown rate of contact pheromone components **12**, **16** and **17**, calculated as ratio of **18** / (**12** + **16** + **17** + **18**) (in blue); control measurements of the water's pH are displayed in red. (**b**) Effect of pH on breakdown of synthetic contact pheromone component **12**, calculated as the ratio of **18** / (**12**+**18**). Circles and boxplots show the breakdown rate of each sample and the distribution of data (minimum, first quartile, median, third quartile, maximum), respectively, at pH 4 and pH 7; medians with the same letter indicate no significant difference in breakdown rates (Kruskal-Wallis test; $P < 0.05$). Note the different scales of the x-axis in subpanels **a** and **b**; **12** = *N*-4-methylvaleroyl-*O*-isobutyroyl-*L*-serine; **18** = *N*-4-methylvaleroyl-*L*-serine (amide), ACN = acetonitrile.

Figure 4.1: **Graphical comparison of total ion chromatograms of a hypothetical case sample (upper trace) and a control sample (lower trace).** (a) A unique compound (green) in the case sample is absent in the control sample. (b) A novel compound (blue) in the case sample is masked – and thus easily overlooked – by a compound (brown) present in both samples. (c) A unique trace compound (red) in the case sample might not be detected.

Figure 4.2: **Phylogeny and comparison of pheromone components (contact & airborne) in widow spiders (Latrodectinae).** (**a**) Previously known pheromone components of *Latrodectus hasselti*,[23] *L. hesperus*,[39] *L. geometricus*,[41] and *Steatoda grossa*:[40] *N*-3-methyl-butyryl-*O*-(*S*)-2-methylbutyryl-L-serine methyl ester (**1**), *N*-3-methylbutanoyl-*O*-isobutyroyl-L-serine methyl ester (**2**), *N*-3-methyl-butyryl-*O*-propionyl-L-serine-methyl ester (**3**), *N*-4-methylvaleroyl-*O*-butyroyl-L-serine (**4**), *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**5**), and *N*-4-methylvaleroyl-*O*-hexanoyl-L-serine (**6**). The contact pheromone components **4–6** of *S. grossa* hydrolyse at the ester bond and give to three airborne mate-attractant pheromone components [butyric acid (**7**), isobutyric acid (**8**), and hexanoic acid (**9**)], whereas the amide *N*-4-methylvaleroyl-L-serine (**10**), as another hydrolysis breakdown product, remains on webs and has no behavioural activity. (**b**) Pheromone components of *Steatoda triangulosa* identified in this study. The contact pheromone components *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**5**), *N*-3-methyl-butyryl-*O*-propionyl-L-serine (**11**), and *N*-3-methyl-butyryl-*O*-butyroyl-L-serine (**12**) hydrolyse at the ester bond and give rise to two airborne mate-attractant pheromone components [butyric acid (**7**) and isobutyric acid (**8**)], whereas *N*-4-methylvaleroyl-L-serine (**10**) and *N*-3-methyl-butyryl-L-serine (**13**)

accumulate on webs. Blue-coloured parts of molecules are phylogenetically conserved, whereas green-coloured parts are unique to *Steatoda*. Orange parts are shared between *Latrodectus* spp. and *S. triangulosa*.

Figure 4.3: **Chromatograms, experimental designs, and behavioural bioassay results.** (a) Total ion chromatogram (TIC) of web extract of female *Steatoda triangulosa* analysed by high-performance liquid chromatography - mass spectrometry. (b) TIC of silyl ester-derivatized web extract of female *S. triangulosa* analysed by gas chromatography - mass spectrometry. (c) Comparative XCMS online Cloud Plots of web extracts of mature and immature female S. triangulosa (depicted by solid and dotted lines, respectively), with circles denoting a >35-fold abundance increase of fragment ions in compounds; the larger the circle, the greater the fold-change of a particular ion. (d) T-rod bioassay apparatus. (e) Effects of female *S. triangulosa* web extract (Exp. 4.1) and contact pheromone component 5 (*N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine) (Exp. 2) on courtship by *S. triangulosa* males. (f) Effects of female *S. triangulosa* web extract (Exp. 3), and a ternary blend of contact pheromone components **5**, **11** (*N*-3-methylbutanoyl-*O*-isobutyroyl-L-serine, and **12** (*N*-3-methylbutanoyl-*O*-butyroyl-L-serine) (Exp. 4), on courtship by *S. triangulosa* males. (g) Effects of contact pheromone components **5**, **11** and **12** presented in ternary combination (Exp. 5), and singly (Exps. 6–8), on courtship by *S. triangulosa* males. (h) Arena olfactometer with prisms carrying test stimuli. (i) Attraction of male *S. triangulosa* to webs of female *S. triangulosa* (Exp. 9), and to synthetic mate-attractant pheromone components **7** (butyric acid) and **8** (isobutyric acid) in arena olfactometers. In each of subpanels e–g, different letters indicate statistical differences between test stimuli across experiments (rank sum test; $p<0.05$). In experiments 9 and 10 (subpanel i), the asterisk (*) indicates a significant preference for the test stimulus (binomial test; $p < 0.05$).

Figure 5.1: **Graphical illustrations of a cobweb and experimental designs.** (a) cobweb depicting the safety (retreat) section (green square) with numerous silk strands, and glue-impregnated prey-capture lines (blue square) anchored to the ground. (b) Pheromone components of female *Steatoda grossa*: three serine ester contact pheromone components [*N*-4-methylvaleroyl-*O*-butyroyl-L-serine (**1**), *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**2**) and *N*-4-methylvaleroyl-*O*-hexoyl-L-serine (**3**)] prompt courtship by males, hydrolyse at the ester bond, and give rise to three corresponding mate-attracting acid pheromone components (red) [butyric (**4**), isobutyric (**5**), hexanoic (**6**)], while the serine amide breakdown product (blue), *N*-4-methylvaleroyl-L-serine (7), remains and accumulates on the web. The rate of the hydrolysis breakdown determines the web's attractiveness to males. (c) Design of experiment 1: Three

female S. grossa build their webs for 48 h on three separate 3-dimensional frames (low-web-density setting); after a 12-day intermission, the same three females built their webs together with 27 other females (high-web-density setting). (d) Design of experiment 2: three females first built their webs in a high-web-density setting, and after a 12-day intermission, built webs in a low-web-density setting. (e) Design of experiment 3: three females built their webs first in a low-web-density setting, and after a 12-day intermission, built webs in the same low-web-density setting, but permeated with synthetic mate-attracting pheromone components (**4**, **5**, **6**) at a concentration equivalent to a high-web-density setting. Pheromone components were formulated in mineral oil and released from 27 Eppendorf vials; during the first exposure, Eppendorf vials contained only plain mineral oil. (f) Web-measurements were taken with a thin metal rod marked in 1-cm intervals[39] by recording the number of silken strands touching the rod in each interval. The rod was placed either vertically 1 cm away from the vertex of the triangular prism in the retreat corner (h_R) and the non-retreat corners (h_1, h_2) of the web, or horizontally at the top of the retreat corner (S_R) and the non-retreat corners (s_1 and s_2) of the triangular prism, pointing to the center of the respective hypothenuses Similar horizontal measurements were taken at the halfway-height point of the lateral edges.

Figure 5.2: **Web adjustments by female false black widow spiders in response to changes in social context.** When groups of three test spiders each (n = 16) first built their webs in a low-web-density setting (‘LWD’; three test spiders only), and then rebuilt their webs in a high-web-density setting (‘HWD’; three test spiders together with 27 further spiders) (see Fig. 1), the groups of test spiders rebuilding their webs produced more silk strands for prey capture and safety, and overall, likely in response to perceived greater competition for prey, and predation risk. Conversely, when groups of three test spiders each (n = 16) first built their webs in a HWD setting, and then rebuilt their webs in a LWD setting, they produced fewer silk strands for prey capture and safety, and overall. Blue dots and red triangles indicate data of experimental replicates, and black squares with whiskers represent the mean and standard error. Web adjustments in each subpanel were statistically significant ($p < 0.001$; GLMM).

Figure 5.3: **Web adjustments by female false black widow spiders in response to synthetic sex pheromone indicating social-context change.** When groups of three test spiders each (n = 16) first built their webs in a low-web-density setting (‘LWD’; three test spiders only), and then rebuilt their webs in a low-web-density setting while sensing synthetic pheromone at a concentration equivalent to a high-web-density setting (‘LWD+Pher’), the groups of test spiders rebuilding their webs produced more silk strands for prey capture and safety, and overall, in response synthetic pheromone indicating greater competition for prey, and predation risk. Blue dots and

red triangles indicate data of experimental replicates, and black squares with whiskers represent the mean and standard error. Web adjustments in each subpanel were statistically significant ($p < 0.001$; GLMM).

Figure 5.4: **Adjustments for mate attraction by female false black widow spiders in response to perceived mate competition.** When groups of three test spiders each (n = 16) first built their webs in a low-web-density setting ('LWD'; three test spiders only), and then (a+b) rebuilt their webs in a high-web-density setting ('HWD'; three test spiders together with 27 further spiders), or (c) rebuilt their webs in a low-web-density setting while sensing synthetic pheromone at a concentration equivalent to a high-web-density setting ('LWD+Pher') (see Fig. 1), the groups of test spiders rebuilding their webs deposited more contact pheromone components [*N*-4-methylvaleroyl-*O*-butyroyl-L-serine (**1**), *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**2**) and *N*-4-methylvaleroyl-*O*-hexoyl-L-serine (**3**)] on their webs (upper row) and accelerated their breakdown to mate-attractant pheromone components (red), [butyric (**4**), isobutyric (**5**), hexanoic (**6**)], essentially enhancing their webs' attractiveness to mate-seeking males. The serine amide breakdown product (blue), N-4-methylvaleroyl-L-serine (**7**), accumulates on the web. The rate of the hydrolysis breakdown determines the web's attractiveness to males. Blue dots and red triangles indicate data of experimental replicates, and black squares with whiskers represent the mean and standard error. Note changes in the amounts of contact pheromone components deposited on webs (Exps. 1-3; each $p < 0.001$, GLMM), and in the breakdown rate of contact pheromone components to mate-attractant pheromone components (Exp. 4; $p = 0.022$, GLMM) in response to perceived mate competition.

Figure 6.1: **Anemotactic attraction of male *Steatoda grossa***. First-choice responses of males to specific test stimuli in Y-tube olfactometer experiments 1 (n = 41), 2 (n = 40), 3 (n = 41) and 4 (n = 20). Numbers in bars indicate the number of males choosing the respective stimulus. One male did not respond in Exp. 2. The asterisk (*) denotes a significant preference for the respective stimulus; χ^2 test; $p < 0.05$.

Figure 6.2: **Occurrence of web reduction by male *Steatoda grossa*.** Proportion of *Steatoda grossa* males engaging in web reduction behavior (element of courtship display) in response to test stimuli. In each of experiments 1 (n = 41), 2 (n = 40), and 3 (n = 41), the asterisk (*) denotes a significant preference for the respective stimulus; χ^2 test; $p < 0.05$.

Figure 6.3: **Web-reduction by male *Steatoda grossa* and likelihood of copulation**. The likelihood of males with functional or dysfunctional spinnerets to copulate with the female they courted increased with increasing time they engaged in web reduction behavior; general linear mixed model, p <

0.001; the line shows the predicted likelihood of copulation in relation to the time spent in web-reduction. Female aggression towards males was not affected by the time males spent web-reducing ($\chi^2 = 0.37$, df = 1, p = 0.54). One male with functional spinnerets and one male with dysfunctional spinnerets was cannibalized by the courted female.

Figure 6.4: **Stridulatory apparatus of a male *Steatoda grossa*.** Scanning electron micrographs show (a) teeth-like structures (the scraper) on the anterior ventrum of the abdomen, and (b) ridges (the file) on the posterior tergum of the prosoma (cephalothorax).

Figure 7.1: **Web acceptance tests.** Mean (+ SE) proportion of time spent by female *Steatoda grossa* on test stimuli. Yellow and blue bars denote virgin and mated females, respectively. In experiments 1-12 (n = 30 for each type of female in each experiment), an asterisk (*) denotes a statistically significant behavioral response to a test stimulus (Mann-Whitney U test, $P < 0.05$).

Figure 7.2: **Egg sac acceptance tests.** Proportions of mated females (Exps. 13, 14, n = 30 each) and virgin females (Exp. 15, n = 30) of *Steatoda grossa* accepting or rejecting their own egg sac or the egg sac of a conspecific female. Blue and yellow bars denote mated and virgin females, respectively. An asterisk (*) indicates a statistically significant behavioral response to a test stimulus (binomial test, $P < 0.05$).

List of Acronyms

CEH =	carboxyl ester hydrolase
GC-EAD =	gas chromatographic-electroantennographic detection
GC-MS =	gas chromatography coupled with mass spectrometry
GLM =	generalised linear model
GLMM =	generalised linear mixed model
HPLC =	high performance liquid chromatography
HPLC-MS/MS =	HPLC with tandem mass spectrometry
kHz =	kilo Herz
NMR =	nuclear magnetic resonance spectroscopy
SEM =	scanning electron micrographs
TIC =	total ion chromatogram

Glossary

Pheromone autodetection:	Ability of females to detect their (conspecific) sex pheromone
Chemical cues:	Passive, biological and environmental stimuli that provide recipients with information.
Kleptoparasite:	An animal that steals food or prey from another animal.
Targeted metabolomics:	Measurement of defined groups of chemically characterised and biochemically annotated metabolites.
Non-targeted metabolomics:	Analysis of all the measurable analytes in a sample including chemical unknowns.
Pheromone:	Intra-specific chemical that benefits both the emitter and the receiver.
Semiochemical:	Message-bearing chemical.
Signals:	Evolved means of actively conveying information and influencing the behaviour of receivers.
Stridulation:	Sound production in animals by rubbing two body parts together
Web reduction behaviour:	Courtship behaviour of some male spiders that bundle up a female’s web while adding their own silk.

Chapter 1: Introduction

"Information is a crucial currency for animals from both a behavioural and evolutionary perspective" (Dall et al. 2005). Information may take the form of a signal or cue (Wyatt 2014). Signals are given by one organism (the sender) and are perceived by, and alter the behaviour of, another organism (the signal recipient) in a way that is adaptive to one or both of the participants (Wilson 1975). Signals are intended information that evolved on the part of the sender and the receiver (Wyatt 2014). Signal modalities may be visual, chemical, acoustic, vibratory, tactile, or multi-modal, with chemical signals likely being the oldest and most prevalent type of signals (Breithaupt and Thiel 2011; Wyatt 2014). Intraspecific chemical signals are termed pheromones, defined as a chemical, or blend of chemicals, released by a signaller that causes a response by conspecific signal recipients (Karlson and Lüscher, 1959; Wyatt 2019). Pheromones used during sexual communication are termed sex pheromones (Ayasse et al. 2001). Cues, in contrast, are unintended information, with their perception evolving only on the part of the receiver (Dall et al. 2005). Cues are any features of the world, animate or inanimate, that can be used to guide future action (Maynard Smith and Harper 1995). For example, chemical cues can reveal the presence of a predator and prompt escape behaviour in prey.

Insects are a long-standing model taxon for studying profound questions in the field of chemical ecology (Ayasse et al. 2001; Ando et al. 2004; Symonds and Elgar 2008; Wyatt 2014). Studying the science of insect scent, the scientific community has elucidated the molecular structure of many pheromones (Ando et al. 2004; Francke and Schulz 2010), revealed their diverse intraspecific functions (Keeling et al. 2004), and explored the gland tissues producing pheromones (Vosshall et al. 1999; Blomquist and Vogt 2003; Ando et al. 2004; Martin et al. 2011). We are beginning to understand the mechanisms of insect olfaction, including the roles of odour-binding and receptor proteins in olfactory receptors (Vosshall et al. 1999). The acquired knowledge has inspired the design of artificial noses (Pelosi et al. 2018) and enabled the application of synthetic pheromones for insect pest detection, monitoring, and control (Cook et al. 2007;

Howse et al. 2013; Kydonieus 2017). Whatever science has discovered in insect chemical ecology remains largely unknown for other taxa including spiders (Symonds and Elgar 2008).

In spiders, olfaction and contact chemoreception are likely the most important sensory modalities of communication (Uhl and Elias 2011; Uhl 2013; Foelix 2015). Mate attraction and assessment (Baruffaldi and Andrade 2015; Fischer et al. 2021), prey location (Jackson and Cross 2015), predator avoidance (Schonewolf et al. 2006), and habitat selection (Johnson et al. 2011), are all mediated by semiochemicals (message-bearing chemicals) (Fischer 2019). Despite the importance of sex pheromones for mate attraction, only 13 sex pheromones of spiders have been identified (Fischer 2019). Not only are the chemical structures of most spider sex pheromones unknown, their biosyntheses and dissemination mechanisms are largely unexplored (Symonds and Elgar 2008; Uhl 2013). The spiders' chemoreceptors are thought to be S-shaped hairs with perforated tips scattered on legs, but there is limited experimental evidence supporting this assumption (Foelix and Chu-Wang 1973; Tichy et al. 2001; Foelix 2015; Ganske and Uhl 2018; Müller et al. 2020). Chemical information is transmitted substrate-borne (contact) or air-borne (volatile) (Uhl and Elias 2011). Substrate-borne transmission requires physical contact by the receiver with the emitting source such as spider web silk (Prouvost et al. 1999; Baruffaldi et al. 2010; Baruffaldi and Andrade 2015; Fischer et al. 2021) or other surfaces (Johnson et al. 2011). In contrast, airborne (volatile) chemical information can be perceived from a distance (Kasumovic and Andrade 2004). Spiders disseminate volatile chemical information from their webs' silk or their body surface (Schulz 2013; Fischer et al. 2021). The large surface area of silk strands in spider webs is believed to facilitate the dissemination of semiochemicals (Watson 1986; Schulz and Toft 1993).

Reviews on chemical communication in spiders (Schulz 1997, 2004; Huber 2005; Gaskett 2007; Uhl and Elias 2011; Schulz 2013; Trabalon 2013; Uhl 2013; Foelix 2015; Fischer 2019) have emphasised different topics. Foelix (2015) focused on morphological and biological perspectives of chemical communication. Huber (2005), Uhl (2013), and Uhl & Elias (2011) highlighted behavioural-biological perspectives and mainly discussed

responses of signal recipients. Gaskett (2007) provided a detailed overview of behavioural studies, and the types of bioassays designed to test for pheromonal communication in select species. Schulz (2004, 2013) focused on the molecular structure of sex pheromones in spiders and their phylogeny. Trabalon (2013) and Schulz (1997) discussed cuticular lipids which function in inter-specific communication rather than in mate attraction. Recently, Fischer (2019) reviewed analytical procedures for spider pheromone identification, described communication signals and environmental cues that are perceived by spiders, and highlighted open questions in spider chemical ecology, some of which are addressed in subheadings 1.1-1.6 below.

1.1 Chemical crypsis of subadult females

Only few studies have investigated pre-copulatory inter-sexual conflicts (Uhl et al. 2015). Mate-seeking adult males of web-building spiders often cohabit and guard the webs of sessile sub-adult (i.e. penultimate instar) females (Jackson 1986; Miller and Miller 1986; Watson 1990, 1991; Anava and Lubin 1993; Dodson and Beck 1993; Eberhard et al. 1993; Miyashita and Hayashi 1996; Fahey and Elgar 1997; Segev et al. 2003; Bel-Venner and Venner 2006; Uhl et al. 2015; Biaggio et al. 2016). If the guarding male succeeds in fending off all rival males and in mating the female, he often gains reproductive fitness due to first male sperm precedence (Watson 1986, 1990, 1991). To optimise his energy expenditure and keep guarding to a minimum, the guarding male would benefit from being able to gauge the latency to a female's maturity mould. The guarded female, in turn, would benefit from mating the fittest male that has been fending off rivals over an extended period of time (Watson 1990, 1991). Kleptoparasitism (Segev et al. 2003; Erez et al. 2005) and diminished risk of sexual cannibalism coupled with maximum life time reproductive fitness (Uhl et al., 2015; Biaggio et al., 2016) are distinct benefits for a cohabiting spider male. For example, males of the black widow *Latrodectus hasselti* chew open the cuticle to the epigyne of sub-adult females and then mate with them, thus avoiding cannibalism (Andrade 1996; Biaggio et al. 2016). Sub-adult females of some spider taxa incur multiple costs from a cohabitating male, including reduced prey capture, kleptoparasitism by the male with deleterious effects on their nutrient intake and

well-being (Watson 1990; Segev et al. 2003; Erez et al. 2005), and curtailed opportunity for mate choice or mate cannibalism, losing nutrient intake from cannibalised males (Buskirk et al. 1984; Uhl et al. 2015; Biaggio et al. 2016). The cues adult male spiders exploit to locate webs of sub-adult females are not understood (Symonds and Elgar 2008; Uhl and Elias 2011). While adult females, or their webs, disseminate sex pheromone that attracts males (Uhl 2013), sub-adult females apparently do not produce sex pheromones (Schulz 2013; Uhl et al. 2015; Fischer 2019). Therefore, males are generally thought to find sub-adult females by chance encounter (Uhl et al. 2015). This assumption, however, lacks empirical studies.

1.2 Origin and identification of contact and volatile sex pheromone components

More than 3000 insect pheromones have been identified (Symonds and Elgar 2008). Using the insects' antennae as an analytical tool to help locate candidate pheromone components in complex analytical samples (Arn et al. 1975) has been instrumental for identifying many of these pheromones, particularly those that occur at trace quantities (e.g., Gries et al. 2021). In contrast, only 12 spider pheromones have been identified, and neither their site of production nor their site of olfactory reception is known (Schulz 2013; Fischer 2019).

Insect pheromones are typically identified using a combination of two primary analytical tools: coupled gas chromatographic-electrographic detection (GC-EAD) analyses and coupled GC-mass spectrometry (MS) (Francke and Schulz 2010; Fischer 2019). GC-EAD analyses of pheromone gland extracts or headspace volatile extracts use insect antennae as biological detectors to locate candidate pheromone components in complex odour samples, whereas GC-MS analyses reveal the mass spectrum of those compounds that have elicited antennal responses and thus are candidate pheromone components. Mass spectra, in turn, provide important analytical information for the identification of compounds. Both GC-EAD and GC-MS analyses require odorants that - based on their chemical characteristics - can be readily gas chromatographed and separated. However, this criterion applies to only a limited number of compounds (Rood

2007). Very polar compounds with e.g., multiple hydroxyl and/or amine groups, such as sugars and amino acids, do not gas chromatograph well and often cannot be analysed by GC-EAD or GC-MS. Yet, most spider pheromones known to date are very polar (Schulz 2013), and thus would not be readily analysable by GC-MS. Instead, spider pheromones should be analysed by coupled high-performance liquid chromatography-mass spectrometry (HPLC-MS), where polar compounds are separated based on their hydrophilicity, electrostaticity or molecular size (Harris 2016). Applying multiple analytical tools for spider pheromone analyses will enhance the probability of finding new spider pheromones.

Unlike insects that typically disseminate pheromones from specific gland tissues (e.g., Ando et al. 2004; Chemnitz et al. 2017), female web-building spiders deposit pheromones on their silken webs (Fischer 2019). Their webs attract males over long distances (Kasumovic and Andrade 2004) and upon contact with the web elicit courtship in males (Scott et al. 2018b), implying the release of mate-attractant pheromone components from the web and the presence of contact pheromone components on the web (Fischer 2019). To date, it is not known whether (*i*) spider pheromones originate from a silk gland, (*ii*) mate-attractant and contact pheromone components are structurally and functionally related, and (*iii*) female spiders can actively modulate the release of mate-attractant pheromone components from their webs.

1.3 Non-targeted metabolomics to aid pheromone identification.

Most pheromones have been identified in insects because their antennae could be used as bio-detectors in electrophysiological recordings to locate candidate pheromone components in complex samples (Roelofs 1984; Symonds and Elgar 2008). However, antennae are conspicuously absent in many animal taxa which might explain the paucity of pheromones identified in these taxa (Wyatt 2014). Thus, spiders have received little attention in chemical ecology research (Symonds and Elgar 2008). There are some 50,000 spider species but only 13 sex pheromones have been identified to date (Symonds and Elgar 2008; Schulz 2013; Fischer 2019), despite ample behavioural evidence for sex pheromonal communication in most spider taxa (Gaskett 2007). Comparative

metabolomics in pheromone identification research compares analytes obtained from animals that were capable (e.g. sexually mature), or not (e.g. sexually immature), of producing pheromone (Fischer 2019). Traditional *targeted* metabolomics compares peaks and focuses on peaks for pheromone identification that are visually unique in one type of analyte (Sramkova et al. 2008; Jerhot et al. 2010; Gillard et al. 2013; Fischer et al. 2021). However, exclusive focus on visually unique peaks may miss pheromone components that co-elute with non-pheromonal compounds or occur at trace quantities.

Non-targeted metabolomics, in contrast, considers *all* detected ions and enables quantitative comparison of ions between samples. This comprehensive approach reduces the probability of erroneously excluding peaks from analyses that are masked by other compounds or occur at trace quantities. *Non-targeted* metabolomics –has been applied, among others, in studies of diet and health (Zhang et al. 2020), sport and exercise (Heaney et al. 2019), host-microbiota (Zhao et al. 2022), drug discovery (Alarcon-Barrera et al. 2022), plant metabolisms (Anzano et al. 2022), organismal responses to environmental toxicants (Liu et al. 2022), and biomarker discovery in disease diagnosis (Baima et al. 2021). *Non-targeted* metabolomics also seems to be a promising analytical tool for pheromone search.

1.4 Recognition of female-female conflict by mate-attractant pheromone components

Female-female conflict has received little attention (Ah-King 2022). There has been a gender bias in selection theory, and research has prioritised males over females in studies of evolutionary selection for secondary sexual traits, such as marked coloration, large size, or striking adornments, possibly because these traits are generally more apparent in males than in females (Ah-King 2022). That competition among females can be an evolutionary force has only recently been acknowledged (Tang-Martínez 2016).

It is now known that females of at least some insect species do sense, and respond to, their own sex pheromone (Holdcraft et al. 2016). For examples, females of the cotton bollworm, *Heliothis armigera,* the corn earworm, *Helicoverpa zea*, and the

Mediterranean flour moth*, Ephestia kuehniella,* all avoid, or disperse from, locations with pheromone-permeated air (Saad and Scott 1981; Trematerra and Battaini 1987). However, the proximate resources for which these females compete have not been empirically studied.

Ecological theory predicts that a complex social context invokes competition for prey and mates, but little is known whether it also invokes predator defense mechanisms in prospective prey (Harari and Steinitz 2013; Clutton-Brock and Huchard 2013). Aggregated animals in a complex social context are more likely than solitary animals to draw the attention of predators (Ayelo et al. 2021).

Female cobweb spiders are ideal models for studying the effects of perceived same-sex competition and risk of predation (Blackledge and Zevenbergen 2007; Fischer et al. 2022). Cobwebs, like other spider webs, have three main functions: prey capture (Foelix 2015), mate attraction (Fischer 2019), and safety from potential predators such as spider-hunting wasps that respond to chemical cues from spider prey (Uma and Weiss 2010, 2012). Whether female spiders can sense, and respond to, their own sex pheromone, and use this ability to reduce prey and mate competition, has never been investigated. Cobwebs, despite their seemingly unorganised appearance, have highly functional architecture to address all the spider's needs. These needs, however, are ever changing. For example, hungry spiders invest more in prey-capture silk than do sated spiders (Blackledge and Zevenbergen 2007). Similarly, spiders in high-web-density settings with perceived competition for prey should invest heavily in silk for prey capture. Furthermore, spiders in high-web-density settings, with vast chemical cues for spider-hunting wasps to exploit (Uma and Weiss 2010), may perceive an increased risk of predation, and thus fortify their webs' safety area. Web adjustment by spiders in response to perceived competition for prey and mates, as well as risk of predation, can be measured by quantifying changes in web characteristics, such as the number of silken strands females produce for prey-capture and safety. Moreover, perceived mate competition can be assessed by quantifying the amount of courtship-inducing contact pheromone components deposited on silk, and by determining the rate of their breakdown to airborne mate-attractant pheromone components.

1.5 Pheromones of spider males

In sexual communication systems of web-building spiders, females as the signalling sex attract males. However, male spiders too produce chemical signals during courtship that affect the behaviour of females (Fischer 2019). Functional roles assigned to male-produced pheromones include aphrodisiac (Xiao et al. 2010) anti-aphrodisiac (Watson 1986), reduction of female aggression (DiRienzo et al. 2019), and induction of female catalepsy (Becker et al. 2005). The aphrodisiac (*Z*)-9-tricosene produced by *Pholcus beijingensis* males reduces the latency to copulation and is the only male spider sex pheromone identified to date (Xiao et al. 2010).

Male courtship also serves to curtail the attractiveness of female webs. Males of *Linyphia litigiosa* and *Latrodectus hesperus* courting on a female's web reduce the web's attractiveness to rival males (Watson 1986; Scott et al. 2015a). This courtship effect, however, seems to be based not exclusively on male pheromones. Courting *L. hesperus* males cut and bundle the female's web by adding their own silk, thus reducing the attractiveness of the female's web (Scott et al. 2018a). In a field experiment, reduced webs with male silk deposition attracted three times fewer males than intact webs, suggesting that web reduction alters, or prevents, emission of silk-borne female pheromones (Scott et al. 2015a), and/or that male pheromone deposited on webs is off-putting to mate-seeking males. As neither experimental (mechanical) removal of half of the web nor addition of male silk to intact webs affected the webs' attractiveness (Scott et al. 2015a), it follows that neither reduction in silk surface area, nor the addition of male silk alone, is sufficient to decrease web attractiveness. Regardless, web reduction helps males reduce male-male conflict during lengthy courtship.

Another courtship function in cannibalistic theridiid spiders is suppression of female aggression or predatory responses. Males of *L. hesperus* that engaged in web reduction elicited fewer aggressive responses from females and induced female quiescence more quickly than did males not exhibiting web reduction behaviour (Scott et al. 2012; DiRienzo et al. 2019). However, whether these effects were caused by male pheromone and/or other signals has yet to be determined.

1.6 'Mine or thine' chemical cues mediate recognition of self and non-self

Pheromones are not the only intra-specific semiochemicals. There are also signature blends that convey the identity of individuals (Wyatt 2014). This phenomenon is particularly well demonstrated in house mice, *Mus musculus* (Wyatt 2014). For individual spiders, it is not known whether they recognize their own signature blend, and the specific blend of individual conspecifics. In other words, it is not known whether spiders discern between 'self' and 'non-self' based on semiochemicals (Fischer 2019). Recognising 'self' would be adaptive to solitary web-building spiders that share a microhabitat. After having been displaced from their webs through a disturbance, spiders would benefit at their return from recognising their own web or egg sac. When threatened, spiders often drop out of their webs to avoid predation (Uma and Weiss 2012). Selecting a web still occupied by a conspecific female may result in conflict or even cannibalism (Wise 2006). Whether displaced theridiids can discern their own webs and conspecific webs is not yet known.

A displaced, previously egg sac-guarding female spider would accrue fitness benefits from recognizing and returning to her own web. Otherwise, her egg sac would remain undefended in the vacated web and be vulnerable to predation or parasitism (Austin 1985). Recognising both her own web and egg sac would be a fail-safe mechanism, ensuring the reproductive fitness of a displaced previously egg sac-guarding female.

1.7 Model spiders

The false black widow spider, *Steatoda grossa,* and the triangulate cobweb spider, *S. triangulosa* (both Araneae: Theridiidae), are the model organisms of my thesis. Using *S. grossa,* I investigated outstanding questions related to spider chemical and communication ecology described above. *Steatoda grossa* and *S. triangulosa* are globally invasive synanthropic spiders (Bellmann 2010; Bradley 2012). Females remain sessile on their cobwebs, where they live, hunt and mate. Unlike females, adult males abandon the

web-dwelling lifestyle in search for mating partners guided by pheromone emanating from female webs (Scott et al. 2018b). Upon arrival on a female's web, contact chemical signals deposited on the web by the female spider elicited courtship behaviour by the male that - as part of the courtship behaviour - bundles up the web by adding his own silk (Knoflach 2004). Male *S. grossa* are polygynous, and females are polyandrous with first sperm precedence (Gwinner-Hanke 1970; Scott et al. 2018b; Welzenberger 2018). *Steatoda* spp. are close relatives of the comparably well studied *Latrodectus* spp. (Garb and Hayashi 2013; Liu et al. 2016; Wheeler et al. 2017). Female-produced contact pheromone components of *Latrodectus* spp. that prompt courtship by males have been described as *N*-3-methylbutanoyl-*O*-(*S*)-2-methylbutyryl-L-serine methyl ester for *L. hasselti* (Jerhot et al. 2010), and *N*-3-methylbutanoyl-*O*-methylpropanoyl-L-serine methyl ester for *L. hesperus* (Scott et al. 2015b). No pheromone was known for any *Steatoda* spp. As shown in many other species, male *S. grossa* cohabit with subadult females (Jackson 1986).

1.8 Overview of research chapters

In chapter 2 (Research Chapter 1) of my thesis, I tested the hypothesis that subadult *S. grossa* females are chemically cryptic to males. I tested the hypothesis in laboratory experiments by presenting adult males with binary choices between different types of webs (e.g., webs of adult virgin females, sub-adult females or sub-adult males), and methanol extracts of these webs. Males spent more time on webs, or web extracts, of adult virgin females than on webs or web extracts of any other type. Most males (95%) also displayed courtship only on webs, or web extracts, of adult virgin females. These data demonstrate apparent semiochemical crypsis of sub-adult females, or their webs, to mate-seeking adult males that seem to find sub-adult females by chance encounter. This crypsis is likely adaptive to sub-adult females that are in sexual conflict with adult males cohabiting their webs.

In chapter 3 (Research Chapter 2), I report three new contact pheromone components produced by female *S. grossa*: *N*-4-methylvaleroyl-*O*-butyroyl-L-serine, *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine and *N*-4-methylvaleroyl-*O*-hexanoyl-L-serine.

The compounds originate from the posterior aggregate silk gland, induce courtship by males, and web pH-dependently hydrolyse at the carboxylic-ester bond, giving rise to three corresponding carboxylic acids that attract males. A carboxyl ester hydrolase (CEH) enzyme is present on webs and likely mediates the functional transition of contact sex pheromone components to the carboxylic acid mate-attractant pheromone components. As CEH activity is pH-dependent, and female spiders can manipulate their silk's pH, they might also actively adjust their webs' attractiveness.

In chapter 4 (Research chapter 3), I investigated the sex pheromone of *Steatoda triangulosa* using non-targeted metabolomics – together with high-performance liquid chromatography-mass spectrometry (HPLC-MS), gas chromatography-MS, and behavioural bioassays. A ternary blend of three contact pheromone components (*N*-4-methylvaleroyl-*O*-isobutyryl-L-serine, *N*-3-methyl-butyryl-*O*-propionyl-L-serine, and *N*-3-methyl-butyryl-*O*-butyroyl-L-serine) was identified. Two of the three pheromone components were only discovered using non-targeted metabolomics. Hydrolysis of the contact pheromone components at the ester bond gave rise to two mate-attractant pheromone components (butyric acid and isobutyric acid) which attracted male *S. triangulosa*.

In chapter 5 (Research Chapter 4), I tested the hypotheses that female *S. grossa* sense their social context and alleviate adverse effects related to intra-sexual competition and to predation risk. Females exposed to synthetic sex pheromone adjusted their webs, indicating perception of intra-sexual competition via 'autodetection' of their conspecific sex pheromone. When females sequentially built their webs in settings of low and high intra-sexual competition and predation risk (3 and 30 webs, respectively), they adjusted their webs to increase prey capture and lower predation risk. In 30-web settings with strong mate competition, females deposited more contact pheromone components on their webs and accelerated their breakdown to mate-attractant pheromone components, essentially increasing their webs' attractiveness to mate-seeking males. All data combined show that female *S. grossa* respond to sexual, social and natural selection pressures originating from intra-sexual conflict.

In chapter 6 (Research Chapter 5), I investigated functional roles of courtship behaviour by *S. grossa* males. I tested the hypotheses that (1) web reduction by males renders webs less attractive to rival males; (2) deposition of silk by courting males has an inter-sexual (male-female) signal function that enhances their likelihood of copulation; and (3) stridulatory sound is a courtship signal of males. Testing anemotactic attraction of males in Y-tube olfactometer experiments revealed that reduced webs (indicative of a mated female) and intact webs (indicative of a virgin female) were equally attractive to males. Recording courtship behaviour of males with either functional (silk-releasing) spinnerets, or spinnerets experimentally occluded, on the web of virgin females showed that males with functional spinnerets were more likely to copulate with the female they courted. Although males possess the stridulatory apparatus to produce courtship sound, they did not stridulate when courting or copulating on the web of females. The data support the conclusion that web reduction behaviour of *S. grossa* males in their invaded North American range has no long-range effect on mate-seeking males. Instead, web reduction behaviour has an inter-sexual signalling function that seems to be linked to functional spinnerets of the courting male. The signal produced by a male likely entails a volatile silk-borne pheromone but may also embody a gauge of his endurance (the amount of time he engages in web reduction causing web vibrations).

In last chapter 7 (Research Chapter 6), I investigated whether female *S. grossa* differentiate between their own silk and silk of conspecifics. I tested the hypotheses (H1, H2) that females prefer their own webs, and the chemical extract of their own webs, to those of conspecifics, and (H3) that mated females discern their own egg sacs and that of conspecifics. In choice bioassays, females indiscriminately accepted both their own webs and egg sacs and those of conspecific females, although they chose extracts of webs based on their chemical cues. The females' indiscriminate responses to webs or egg sacs are likely due to a lack of selection pressure to reject webs, or egg sacs, of conspecific females.

1.9 References

Ah-King M (2022) The history of sexual selection research provides insights as to why females are still understudied. Nat Commun 13:6976. https://doi.org/10.1038/s41467-022-34770-z

Alarcon-Barrera JC, Kostidis S, Ondo-Mendez A, Giera M (2022) Recent advances in metabolomics analysis for early drug development. Drug Discovery Today 27:1763–1773. https://doi.org/10.1016/j.drudis.2022.02.018

Anava A, Lubin Y (1993) Presence of gender cues in the web of a widow spider, *Latrodectus revivensis*, and a descrition of courtship behaviour. Bulletin of the British Arachnological Society 9:119–122

Ando T, Inomata S, Yamamoto M (2004) Lepidopteran sex pheromones. Topics in Current Chemistry 239:51–96. https://doi.org/10.1007/b95449

Andrade MCB (1996) Sexual selection for male sacrifice in the Australian Redback Spider. Science 271:70–72. https://doi.org/10.1126/science.271.5245.70

Anzano A, Bonanomi G, Mazzoleni S, Lanzotti V (2022) Plant metabolomics in biotic and abiotic stress: a critical overview. Phytochem Rev 21:503–524. https://doi.org/10.1007/s11101-021-09786-w

Arn H, Städler E, Rauscher S (1975) The electroantennographic detector—a selective and sensitive tool in the gas chromatographic analysis of insect pheromones. Z Naturforsch 30c:722–725

Austin AD (1985) The function of spider egg sacs in relation to parasitoids and predators, with special reference to the australian fauna. Journal of Natural History 19:359–376. https://doi.org/10.1080/00222938500770261

Ayasse M, Paxton RJ, Tengö J (2001) Mating behavior and chemical communication in the order hymenoptera. Annual Review of Ecology, Evolution, and Systematics 46:31–78. https://doi.org/10.1063/1.1420510

Ayelo PM, Pirk CWW, Yusuf AA, et al (2021) Exploring the kairomone-based foraging behaviour of natural enemies to enhance biological control: a review. Front Ecol Evol 9:641974. https://doi.org/10.3389/fevo.2021.641974

Baima G, Iaderosa G, Citterio F, et al (2021) Salivary metabolomics for the diagnosis of periodontal diseases: a systematic review with methodological quality assessment. Metabolomics 17:1. https://doi.org/10.1007/s11306-020-01754-3

Baruffaldi L, Andrade MCB (2015) Contact pheromones mediate male preference in black widow spiders: avoidance of hungry sexual cannibals? Animal Behaviour 102:25–32. https://doi.org/10.1016/j.anbehav.2015.01.007

Baruffaldi L, Costa FG, Rodríguez A, González A (2010) Chemical communication in *Schizocosa malitiosa*: Evidence of a female contact sex pheromone and persistence in the field. Journal of Chemical Ecology 36:759–767. https://doi.org/10.1007/s10886-010-9819-x

Becker E, Riechert S, Singer F (2005) Male induction of female quiescence/catalepsis during courtship in the spider, *Agelenopsis aperta*. Behaviour 142:57–70. https://doi.org/10.1163/1568539053627767

Bellmann H (2010) Der Kosmos-Spinnenführer. Kosmos, Stuttgart, Germany

Bel-Venner MC, Venner S (2006) Mate-guarding strategies and male competitive ability in an orb-weaving spider: results from a field study. Animal Behaviour 71:1315–1322. https://doi.org/10.1016/j.anbehav.2005.08.010

Biaggio MD, Sandomirsky I, Lubin Y, et al (2016) Copulation with immature females increases male fitness in cannibalistic widow spiders. Biology Letters 12:1–5

Blackledge TA, Zevenbergen JM (2007) Condition-dependent spider web architecture in the western black widow, *Latrodectus hesperus*. Animal Behaviour 73:855–864. https://doi.org/10.1016/j.anbehav.2006.10.014

Blomquist GJ, Vogt RG (eds) (2003) Insect Pheromone Biochemistry and Molecular Biology. Academic Press, New York

Bradley RA (2012) Common Spiders of North America. University of California Press

Breithaupt T, Thiel M (2011) Chemical communication in Crustaceans. Springer Science+Business Media, New York

Buskirk R, Frohlich C, Ross K (1984) The natural selection of sexual cannibalism. The American Naturalist 123:612–625. https://doi.org/10.1086/284227

Chemnitz J, Bagrii N, Ayasse M, Steiger S (2017) Variation in sex pheromone emission does not reflect immunocompetence but affects attractiveness of male burying beetles—a combination of laboratory and field experiments. Science of Nature 104:. https://doi.org/10.1007/s00114-017-1473-5

Clutton-Brock TH, Huchard E (2013) Social competition and selection in males and females. Phil Trans R Soc B 368:20130074. https://doi.org/10.1098/rstb.2013.0074

Cook SM, Khan ZR, Pickett JA (2007) The use of push-pull strategies in integrated pest management. Annual Review of Entomology 52:375–400. https://doi.org/10.1146/annurev.ento.52.110405.091407

Dall SRX, Giraldeau L-A, Olsson O, et al (2005) Information and its use by animals in evolutionary ecology. Trends in Ecology and Evolution 20:187–193. https://doi.org/10.1016/j.tree.2005.01.010

DiRienzo N, Bradley CT, Smith CA, Dornhaus A (2019) Bringing down the house: male widow spiders reduce the webs of aggressive females more. Behavioral Ecology and Sociobiology 73:1–10. https://doi.org/10.1007/s00265-018-2618-z

Dodson GN, Beck MW (1993) Pre-copulatory guarding of penultimate females by male crab spiders, *Misumenoides formosipes*. Animal Behaviour 46:951–959. https://doi.org/10.1006/anbe.1993.1276

Eberhard WG, Guzman-Gomez S, Catley KM (1993) Correlation between spermathecal morphology and mating systems in spiders. Biological Journal of the Linnean Society 50:197–209. https://doi.org/10.1111/j.1095-8312.1993.tb00926.x

Erez T, Schneider JM, Lubin Y (2005) Is male cohabitation costly for females of the spider *Stegodyphus lineatus* (Eresidae)? Ethology 111:693–704. https://doi.org/10.1111/j.1439-0310.2005.01090.x

Fahey BF, Elgar MA (1997) Sexual cohabitation as mate-guarding in the leaf-curling spider *Phonognatha graeffei* Keyserling (Araneoidae, Araneae). Behavioral Ecology and Sociobiology 40:127–133

Fischer A (2019) Chemical communication in spiders - a methodological review. Journal of Arachnology 47:1–27

Fischer A, Gries R, Alamsetti SK, et al (2022) Origin, structure and functional transition of sex pheromone components in a false widow spider. Commun Biol 5:1156. https://doi.org/10.1038/s42003-022-04072-7
Fischer A, Schulz S, Ayasse M, Uhl G (2021) Pheromone communication among sexes of the garden cross spider *Araneus diadematus*. The Science of Nature 108:1–11. https://doi.org/10.1007/s00114-021-01747-9
Foelix R (2015) Biologie der Spinnen. Chimaira, Frankfurt am Main
Foelix RF, Chu-Wang I-W (1973) The morphology of spider sensilla II. Chemoreceptors. Tissue & Cell 5:461–478. https://doi.org/10.1016/S0040-8166(73)80038-2
Francke W, Schulz S (2010) Pheromones of Terrestrial Invertebrates. In: Mander L, Liu H-W (eds) Comprehensive Natural Products II. Oxford, pp 153–223
Ganske A-S, Uhl G (2018) The sensory equipment of a spider – A morphological survey of different sensillum types in both sexes of *Argiope bruennichi* (Araneae, Araneidae). Arthropod Structure & Development 47:144–161. https://doi.org/10.1016/j.asd.2018.01.001
Garb JE, Hayashi CY (2013) Molecular evolution of alpha-latrotoxin, the exceptionally potent vertebrate neurotoxin in black widow spider venom. Molecular Biology and Evolution 30:999–1014. https://doi.org/10.1093/molbev/mst011
Gaskett AC (2007) Spider sex pheromones: emission, reception, structures, and functions. Biological Reviews 82:27–48. https://doi.org/10.1111/j.1469-185X.2006.00002.x
Gillard J, Frenkel J, Devos V, et al (2013) Metabolomics enables the structure elucidation of a diatom sex pheromone. Angewandte Chemie - International Edition 52:854–857. https://doi.org/10.1002/anie.201208175
Gries R, Alamsetti SK, van Herk WG, et al (2021) Limoniic acid - Major component of the sex pheromones of the click beetles *Limonius canus* and *L. californicus*. Journal of Chemical Ecology 47:123–133. https://doi.org/10.1007/s10886-020-01241-y
Gwinner-Hanke H (1970) Zum Verhalten zweier stridulierender Spinnen *Steatoda bipunctata* Linne und *Teutana grossa* Koch (Theridiidae, Araneae), unter besonderer Berücksichtigung des Fortpflanzungsverhaltens. Ethology 27:649–678. https://doi.org/10.1111/j.1439-0310.1970.tb01893.x
Harari AR, Steinitz H (2013) The evolution of female sex pheromones. Current Zoology. https://doi.org/10.1093/czoolo/59.4.569
Harris DC (2016) Quantitative chemical analysis. W. H. Freeman and Company, New York
Heaney LM, Deighton K, Suzuki T (2019) Non-targeted metabolomics in sport and exercise science. Journal of Sports Sciences 37:959–967. https://doi.org/10.1080/02640414.2017.1305122
Holdcraft R, Rodriguez-Saona C, Stelinski LL (2016) Pheromone autodetection: Evidence and implications. Insects. https://doi.org/10.3390/insects7020017
Howse PE, Stevens IDR, Jones OT (2013) Insect pheromones and their use in pest management. Springer Science+Business Media
Huber BA (2005) Sexual selection research on spiders: progress and biases. Biological Reviews 80:363–385. https://doi.org/10.1017/s1464793104006700

Jackson RR (1986) Cohabitation of males and juvenile females: a prevalent mating tactic of spiders. Journal of Natural History 20:1193–1210. https://doi.org/10.1080/00222938600770791
Jackson RR., Cross FR (2015) Mosquito-terminator spiders and the meaning of predatory specialization. Journal of Arachnology 43:123–142. https://doi.org/10.1636/V15-28
Jerhot E, Stoltz JA, Andrade MCB, Schulz S (2010) Acylated serine derivatives: A unique class of arthropod pheromones of the Australian Redback spider, *Latrodectus hasselti*. Angewandte Chemie 49:2037–2040. https://doi.org/10.1002/anie.200906312
Johnson A, Revis O, Johnson JC (2011) Chemical prey cues influence the urban microhabitat preferences of Western black widow spiders, *Latrodectus hesperus*. Journal of Arachnology 39:449–453. https://doi.org/10.1636/Hi11-19.1
Kasumovic MM, Andrade MCB (2004) Discrimination of airborne pheromones by mate-searching male western black widow spiders (*Latrodectus hesperus*): species-and population-specific responses. Canadian Journal of Zoology 82:1027–1034. https://doi.org/10.1139/z04-081
Keeling CI, Plettner E, Slessor KN (2004) Hymenopteran Semiochemicals. Topics in Current Chemistry 239:133–177. https://doi.org/10.1007/b95452
Knoflach B (2004) Diversity in the copulatory behaviour of comb-footed spiders (Araneae, Theridiidae). Denisia 12:161–256
Kydonieus AF (2017) Insect suppression with controlled release pheromone systems: volume I. CRC Press
Liu J, May-Collado LJ, Pekár S, Agnarsson I (2016) A revised and dated phylogeny of cobweb spiders (Araneae, Araneoidea, Theridiidae): A predatory cretaceous lineage diversifying in the era of the ants (Hymenoptera, Formicidae). Molecular Phylogenetics and Evolution 94:658–675. https://doi.org/10.1016/j.ympev.2015.09.023
Liu L, Wu Q, Miao X, et al (2022) Study on toxicity effects of environmental pollutants based on metabolomics: A review. Chemosphere 286:131815. https://doi.org/10.1016/j.chemosphere.2021.131815
Martin JP, Beyerlein A, Dacks AM, et al (2011) The neurobiology of insect olfaction: Sensory processing in a comparative context. Progress in Neurobiology 95:427–447. https://doi.org/10.1016/j.pneurobio.2011.09.007
Maynard Smith J, Harper DGC (1995) Animal signals: models and terminology. Journal of Theoretical Biology 177:305–311. https://doi.org/10.1006/jtbi.1995.0248
Miller GL, Miller PR (1986) Pre-courtship cohabitation of mature male and penultimate female *Geolycosa turricola* (Araneae, Lycosidae). Journal of Arachnology 14:133–134
Miyashita T, Hayashi H (1996) Volatile chemical cue elicits mating behavior of cohabiting males of *Nephila clavata*. Journal of Arachnology 24:9–15
Müller CHG, Ganske AS, Uhl G (2020) Ultrastructure of chemosensory tarsal tip-pore sensilla of *Argiope* spp. Audouin, 1826 (Chelicerata: Araneae: Araneidae). Journal of Morphology 281:1634–1659. https://doi.org/10.1002/jmor.21276
Pelosi P, Zhu J, Knoll W (2018) From gas sensors to biomimetic artificial noses. Chemosensors 6:1–16. https://doi.org/10.3390/chemosensors6030032

Prouvost O, Trabalon M, Papke MD, Schulz S (1999) Contact sex signals on web and cuticle of Tegenaria atrica (Araneae, Agelenidae). Archives of Insect Biochemistry and Physiology 40:194–202. https://doi.org/10.1002/(SICI)1520-6327(1999)40:4<194::AID-ARCH4>3.0.CO;2-P
Roelofs WL (1984) Electroantennogram assays: rapid and convenient screening procedures for pheromones. In: Hummel HE, Miller TA (eds) Techniques in Pheromone Research. Springer New York, pp 131–159
Rood D (2007) The troubleshooting and maintenance guide for gas chromatographers. WILEY-VCH Verlag, Weinheim
Saad A, Scott D (1981) Repellency of pheromones released by females of *Heliothis armigera* and *H. zea* to females of both species. Entomologia Experimentalis et Applicata 30:123–127. https://doi.org/10.1111/j.1570-7458.1981.tb03085.x
Schonewolf KW, Bell R, Rypstra AL, Persons MH (2006) Field evidence of an airborne enemy-avoidance kairomone in wolf spiders. Journal of Chemical Ecology 32:1565–1576. https://doi.org/10.1007/s10886-006-9070-7
Schulz S (2013) Spider pheromones - a structural perspective. Journal of Chemical Ecology 39:1–14. https://doi.org/10.1007/s10886-012-0231-6
Schulz S (1997) The chemistry of spider toxins and spider silk. Angewandte Chemie 36:314–326. https://doi.org/10.1002/anie.199703141
Schulz S (2004) Semiochemistry of spiders. In: Carde RT, Millar JG (eds) Advances in Insect Chemical Ecology. Cambridge University Press, pp 110–150
Schulz S, Toft S (1993) Identification of a sex pheromone from a spider. Science 260:1635–1637. https://doi.org/10.1128/JVI.79.10.5933
Scott C, Anderson AG, Andrade MCB (2018a) A review of the mechanisms and functional roles of male silk use in spider courtship and mating. Journal of Arachnology 46:173–206
Scott C, Gerak C, McCann S, Gries G (2018b) The role of silk in courtship and chemical communication of the false widow spider, *Steatoda grossa* (Araneae: Theridiidae). Journal of Ethology 36:191–197. https://doi.org/10.1007/s10164-017-0539-3
Scott C, Kirk D, McCann S, Gries G (2015a) Web reduction by courting male black widows renders pheromone-emitting females' webs less attractive to rival males. Animal Behaviour 107:71–78. https://doi.org/10.1016/j.anbehav.2015.06.009
Scott C, McCann S, Gries R, et al (2015b) N-3-Methylbutanoyl-O-methylpropanoyl-L-serine Methyl Ester – Pheromone Component of Western Black Widow Females. Journal of Chemical Ecology 41:465–472. https://doi.org/10.1007/s10886-015-0582-x
Scott C, Vibert S, Gries G (2012) Evidence that web reduction by western black widow males functions in sexual communication. The Canadian Entomologist 144:672–678. https://doi.org/10.4039/tce.2012.56
Segev O, Ziv M, Lubin Y (2003) The male mating system in a desert widow spider. Journal of Arachnology 31:379–393. https://doi.org/10.1636/s01-101
Sramkova A, Schulz C, Twele R, et al (2008) Fertility signals in the bumblebee *Bombus terrestris* (Hymenoptera: Apidae). Naturwissenschaften 95:515–522. https://doi.org/10.1007/s00114-008-0353-4

Symonds MRE, Elgar MA (2008) The evolution of pheromone diversity. Trends in Ecology and Evolution 23:220–228. https://doi.org/10.1016/j.tree.2007.11.009

Tang-Martínez Z (2016) Rethinking bateman's principles: challenging persistent myths of sexually reluctant females and promiscuous males. The Journal of Sex Research 53:532–559. https://doi.org/10.1080/00224499.2016.1150938

Tichy H, Gingl E, Ehn R, et al (2001) Female sex pheromone of a wandering spider (*Cupiennius salei*): Identification and sensory reception. Journal of Comparative Physiology - A 187:75–78. https://doi.org/10.1007/s003590000175

Trabalon M (2013) Chemical communication and contact cuticular compounds in spiders. In: Ecophysiology. Springer, Heidelberg, pp 125–140

Trematerra P, Battaini F (1987) Control of *Ephestia kuehniella* Zeller by mass-trapping. Journal of Applied Entomology 104:336–340

Uhl G (2013) Spider olfaction: attracting, detecting, luring and avoiding. In: Nentwig W (ed) Spider Ecophysiology. Springer, Heidelberg, pp 141–157

Uhl G, Elias D (2011) Communication. In: Herberstein ME (ed) Spider Behaviour. Cambridge University Press, Cambridge, pp 127–189

Uhl G, Zimmer SM, Renner D, Schneider JM (2015) Exploiting a moment of weakness: male spiders escape sexual cannibalism by copulating with moulting females. Scientific Reports 5:16928. https://doi.org/10.1038/srep16928

Uma DB, Weiss MR (2010) Chemical mediation of prey recognition by spider-hunting wasps. Ethology 116:85–95. https://doi.org/10.1111/j.1439-0310.2009.01715.x

Uma DB, Weiss MR (2012) Flee or fight: ontogenetic changes in the behavior of cobweb spiders in encounters with spider-hunting wasps. Environmental Entomology 41:1474–1480. https://doi.org/10.1603/EN12126

Vosshall LB, Amrein H, Morozov PS, et al (1999) A spatial map of olfactory receptor expression in the *Drosophila* antenna. Cell 96:725–736. https://doi.org/10.1016/S0092-8674(00)80582-6

Watson PJ (1986) Transmission of a female sex pheromone thwarted by males in the spider *Linyphia litigiosa* (Linyphiidae). Science 233:219–221

Watson PJ (1990) Female-enhanced male competition determines the first mate and principal sire in the spider *Linyphia litigiosa* (Linyphiidae). Behavioral Ecology and Sociobiology 26:77–90. https://doi.org/10.1007/BF00171577

Watson PJ (1991) Multiple paternity and first mate sperm precedence in the Sierra Dome Spider, *Linyphia litigiosa* Keyserling (Linyphiidae). Animal Behaviour 41:135–148

Welzenberger R (2018) Paarungsverhalten und Fortpflanzungssystem der kosmopolitischen Kugelspinne *Steatoda grossa* (Araneae , Theridiidae). Leopold-Franzens-Universität Innsbruck

Wheeler WC, Coddington JA, Crowley LM, et al (2017) The spider tree of life: Phylogeny of araneae based on target-gene analyses from an extensive taxon sampling. Cladistics 33:574–616. https://doi.org/10.1111/cla.12182

Wilson EO (1975) Sociobiology: The New Synthesis. Harvard University Press, Cambridge

Wise DH (2006) Cannibalism, food limitation, intraspecific competition, and the regulation of spider populations. Annual Review of Entomology 51:441–465. https://doi.org/10.1146/annurev.ento.51.110104.150947

Wyatt TD (2014) Pheromones and animal behavior, 2nd edn. Cambridge University Press, Cambridge
Xiao Y-H, Zhang J-X, Li S-Q (2010) Male-specific (Z)-9-tricosene stimulates female mating behaviour in the spider *Pholcus beijingensis*. Proceedings of the Royal Society B 277:3009–3018. https://doi.org/10.1098/rspb.2010.0763
Zhang X, Li Q, Xu Z, Dou J (2020) Mass spectrometry-based metabolomics in health and medical science: a systematic review. RSC Adv 10:3092–3104. https://doi.org/10.1039/C9RA08985C
Zhao X, Jiang L, Fang X, et al (2022) Host-microbiota interaction-mediated resistance to inflammatory bowel disease in pigs. Microbiome 10:115. https://doi.org/10.1186/s40168-022-01303-1

Chapter 2: Dodging sexual conflict? – Sub-adult females of a web-building spider stay cryptic to mate-seeking adult males[1]

[1]The corresponding manuscript has been published in *Ethology* (Volume 124, Issue 11, pages 838–843; 2018), with the following authors: Andreas Fischer, Yerin Lee, Jordan Stewart & Gerhard Gries

2.1 Abstract

Adult males of web-building spiders often cohabit the webs of sessile sub-adult (i.e., penultimate instar) females and mate with them as they moult to adults. Often, males accrue benefits from this cohabitation (kleptoparasitism, avoidance of cannibalism, potential polygamy), whereas sub-adult females may either accrue benefits or incur costs such as curtailed opportunity for mate choice or mate cannibalism. Working with the false black widow spider, *Steatoda grossa*, we tested the hypothesis that webs of sub-adult females, unlike those of virgin adult females, lack sex attractant pheromone that mate-seeking males could detect and exploit for mate location. We tested our hypothesis in laboratory experiments by presenting adult males with binary choices between different types of webs (e.g., webs of adult virgin females, sub-adult females or sub-adult males), and methanol extracts of these webs. Males spent more time on webs, or web extracts, of adult virgin females than on webs or web extracts of any other type. Most males (95%) also displayed courtship only on webs, or web extracts, of adult virgin females. Our data demonstrate apparent semiochemical crypsis of sub-adult females or their webs to mate-seeking adult males that seem to find sub-adult females by chance encounter. This crypsis is likely adaptive to sub-adult females that are in sexual conflict with adult males cohabiting their webs.

Keywords: Sexual selection, intersexual conflict, sex pheromone, male cohabitation, sub-adult cue, *Steatoda grossa*

2.2 Introduction

Sexual conflict, or sexual antagonism, occurs when males and females have conflicting optimal reproductive strategies. A strategy optimal for one sex to maximize reproductive fitness may be suboptimal for the other (Chapman, Arnqvist, Bangham, & Rowe, 2003). Sexual conflict between males and females has been studied with focus on mating frequency (Eberle & Kappeler, 2004), relative parental effort (Engel et al., 2016), sexual cannibalism (Wise, 2006), female re-mating behaviour (Arnqvist & Nilsson, 2000), and reproductive barriers (Gavrilets, 2000). Yet, only few studies have investigated pre-copulatory inter-sexual conflicts (Uhl, Zimmer, Renner, & Schneider, 2015). For example, males of the butterfly *Heliconius charithonia* sense the sex of a female pupa based on pupa-derived monoterpenes and then guard that pupa until the adult female butterfly ecloses (Estrada, Yildizhan, Schulz, & Gilbert, 2010).

Similarly, mate-seeking adult males of web-building spiders often cohabit the webs of sessile sub-adult (i.e., penultimate instar) females (Anava & Lubin, 1993; Bel-Venner & Venner, 2006; Biaggio, Sandomirsky, Lubin, Harari, & Andrade, 2016; Dodson & Beck, 1993; Eberhard, Guzman-Gomez, & Catley, 1993; Fahey & Elgar, 1997; Jackson, 1986; Miller & Miller, 1986; Miyashita & Hayashi, 1996; Segev, Ziv, & Lubin, 2003; Uhl et al., 2015; Watson, 1990, 1991). In this context, sub-adult females of the linyphiid spider *Neriene litigiosa* have been particularly well studied (Watson, 1986, 1990, 1991). One to five days prior to their maturity moult, females produce an honest signal (Watson, 1990) that elicits web cohabiting and guarding by a male. If the guarding male succeeds in fending off all rival males and in mating the female, he often (70%) gains reproductive fitness due to first male sperm precedence. To optimize his energy expenditure and keep guarding to a minimum, the guarding male would benefit from being able to gauge the latency to a female's maturity mold. The guarded female, in turn, would benefit from mating the fittest male that has been fending off rivals over an extended period of time (Watson, 1990, 1991). Interestingly, virgin female *N. litigiosa* commence production of sex attractant pheromone only 7–10 days post their maturity mold (Watson, 1986; Schulz, 2013), possibly as a fail-safe mechanism to ultimately ensure mate attraction.

Kleptoparasitism (Erez, Schneider, & Lubin, 2005; Segev et al., 2003) and diminished risk of sexual cannibalism coupled with maximum life time reproductive fitness (Uhl et al., 2015; Biaggio et al., 2016) are distinct benefits for a cohabiting spider male. For example, males of the araneid *Argiope bruennichi*, often cohabit with sub-adult females and mate with them as they moult to adults, thus greatly reducing the risk of sexual cannibalism (Uhl et al., 2015). Similarly, males of the black widow *Latrodectus hasselti* chew open the cuticle to the epigyne of sub-adult females and then mate with them, thus avoiding cannibalism (Andrade, 1996; Biaggio et al., 2016). However, sexual cannibalism of males can also be considered a trade-off between a male's paternal investment (self-sacrifice to nutrient-provision the female) and increased life time reproductive fitness (Buskirk, Frohlich, & Ross, 1984).

Sub-adult females of some spider taxa incur multiple costs from a cohabitating male, including reduced prey capture, kleptoparasitism by the male with deleterious effects on their nutrient intake and well-being (Erez, Schneider, & Lubin, 2005; Segev et al., 2003; Watson, 1990), and curtailed opportunity for mate choice or mate cannibalism, losing nutrient intake from cannibalised males (Biaggio et al., 2016; Buskirk et al., 1984; Uhl et al., 2015).

The cues adult male spiders exploit to locate webs of sub-adult females are not understood (Symonds & Elgar, 2008; Uhl & Elias, 2011). While adult females, or their webs, disseminate sex pheromone that attracts males (Uhl, 2013 and references cited therein), sub-adult females apparently do not produce sex pheromones (Schulz, 2013; Uhl et al., 2015). Therefore, males are generally thought to find sub-adult females by chance encounter (Uhl et al., 2015). Conversely, sub-adult females of the wolf spiders *Schizocosa ocreata* and *S. malitiosa* produce silk that is attractive to males (Baruffaldi & Costa, 2010; Roberts & Uetz, 2005). Indeed, sub-adult females and freshly molted virgin adult females of *S. ocreata* produce silk that elicits similar responses by males (Roberts & Uetz, 2005). The males' attraction to both sources of silk is attributed to chemical attractants but silk extracts were not bioassayed in this study. Both the silk (or silk extract) of adult virgin female *S. malitiosia* and – surprisingly – the silk of sub-adult male

S. malitiosia elicit courtship behaviour by males (Baruffaldi & Costa, 2010; Baruffaldi, Costa, Rodríguez, & González, 2010).

The false black widow spider, *Steatoda grossa* (Theridiidae), is a close relative of *L. hasselti* (Garb & Hayashi, 2013) and occurs predominantly indoors (Bellmann, 2010). Thus far, it has been the focus of only a few studies (Braun, 1956; Gerhardt, 1924; Gwinner-Hanke, 1970; Scott, Gerak, McCann, & Gries, 2018). Unlike males of other web-building spiders, males of *S. grossa* build webs, continuously hunt and feed, and thus may live for >1.5 years (Gwinner-Hanke, 1970). Webs of adult female *S. grossa* disseminate a (methanol-extractable) sex pheromone that attracts adult males and that induces courtship (web-wrapping) behaviour in males (Scott et al., 2018). Sexual cannibalism and its direct benefit for females has not yet been intensely studied in *Steatoda spp.,* but facultative sexual cannibalism in *S. grossa* (Knoflach, 2004) may explain field observations of adult males cohabiting the webs of sub-adult females (Jackson, 1986).

The strategy of *S. grossa* males to cohabit the webs of sub-adult females causes sexual conflict in that females suffer losses in reproductive fitness (see above). Therefore, it would be adaptive to sub-adult females not to produce any cues that help males locate them. We tested the hypothesis that webs of sub-adult females, unlike those of virgin adult females, lack any sex attractant pheromone that mate-seeking males could detect and exploit. We tested our hypothesis by recording behavioural responses of adult males to different types of webs (e.g., webs of adult virgin females, sub-adult females or sub-adult males) and extracts of these webs.

2.3 Methods

2.3.1 Experimental Spiders

Experimental spiders were the F1 offspring of mated females collected from hallways of the Burnaby campus of Simon Fraser University (Burnaby, B.C., Canada). Upon hatching, juvenile spiders were housed individually in petri dishes (100 × 20 mm) and provisioned with the vinegar flies *Drosophila melanogaster*. Sub-adult males and

females were kept in different rooms, as sub-adult males invest in maturation, rather than growth, when exposed to female sex pheromone (Cory & Schneider, 2017; Kasumovic & Andrade, 2006). Sub-adult spiders were fed with larvae of the mealworm beetle *Tenebrio molitor*. Each adult female spider was kept in a separate translucent 300-mL plastic cup (Western Family, Canada) maintained at 22°C under a reversed light cycle (12:12 h). Adult males and females were fed with black blow flies, *Phormia regina*. All spiders had access to water in cotton wicks. Water and food were provided once per week.

A total of 150 males (25 in each experiment) was tested (see below). As males build webs and continue to hunt and feed throughout their long life (>1.5 years), the age of males was considered not an experimental variable in our study.

2.3.2 Web experiments

Webs of adult virgin females, sub-adult females and sub-adult males were tested for behavioural responses of adult males (Exps. 1-5; N = 25 each). Spiders were allowed to build webs for three days on wooden triangular prisms (30 × 25 × 22 cm) made of bamboo skewers (GoodCook, CA, USA) (see Scott et al. 2018). Prior to bioassays, the spiders were removed from their webs and the web-bearing frames were placed at either ends of a large T-rod climbing structure. The T-rod consisted of a horizontal beam (72 × 0.44 cm) and a vertical beam (19 × 0.44 cm) held together by labelling tape (3 × 1.9 cm, Fisher Scientific, Ottawa, ON, Canada). Each frame was positioned atop a petri dish in a water-filled tray to prevent the bioassayed adult male, which was introduced on the vertical beam, from escaping the experimental arena that was illuminated by red light. Each bioassay lasted 15 min and was terminated earlier only if the bioassay male fell into the water and was not able to return to the test arena. For each male, we recorded (*i*) the time he spent on each frame, (*ii*) and the time he spent displaying courtship behaviour. Each T-rod and web was used only once, whereas some males were retested in a second experiment with other stimuli. On any one day, the same number of replicates was run for each of the parallel experiments 1-5 and 6-8 (see below).

Experiments 1-3 were designed to test and compare the effects of webs produced by a virgin adult female or a sub-adult female on the responses of males. In each

replicate, we offered an adult male a choice between two frames bearing (*i*) the web of a virgin adult female or no web (Exp. 1), (*ii*) the web of a virgin adult female or the web of a sub-adult female (Exp. 2), and (*iii*) the web of a sub-adult female or no web (Exp. 3). Experiments 4-5 were designed to test and compare the effects of webs produced by a sub-adult female and a sub-adult male. In each replicate, we offered the bioassay male a choice between two frames bearing (*i*) the web of a sub-adult female or the web of a sub-adult male (Exp. 4), and (*ii*) the web of a sub-adult male or no web (Exp. 5).

2.3.3 Web extract experiments

To test the effect of web semiochemicals, in the absence of web silk, on the responses of adult males, each web was extracted for 24 h in 50 µL of methanol (99.9 % HPLC grade, Fisher Chemical, ON, Canada) after which the web was removed. The webs of virgin adult females and sub-adult females were pooled for each of the two groups and then weighed using a Sartorius Secura 125-1S Semi Micro Analytical Balance (Sartorius, Göttingen, Germany). The web weight of an adult virgin female (N = 169) and sub-adult female (N = 104) averaged 0.311 mg and 0.155 mg, respectively. To compensate for this 2-fold mass differential between webs of adult and sub-adult females, possibly resulting in disparate amounts of pheromone, web extracts of sub-adult females were tested at both 1 and 2 web equivalents (see experiments 6, 7 below). For each bioassay, web extract, or the corresponding amount of methanol, was applied to a piece of filter paper (2 cm^2) positioned at the ends of the horizontal beam of a small T-rod climbing structure (horizontal arm: 25 × 0.4 cm, vertical beam 30 × 0.4 cm). Methanol was allowed to evaporate for 1 min prior to the onset of a bioassay. The bioassay protocol was identical to that for the large T-rod bioassay (see above).

Experiments 6-8 were designed to compare the effect of web extracts prepared from webs woven by a virgin adult female, a sub-adult female or a sub-adult male on the responses of males. In each replicate, we offered an adult bioassay male a choice between two pieces of filter paper (see above) that were treated with (*i*) the web extract of a virgin adult female (1 web equivalent) or that of a sub-adult female (1 web equivalent) (Exp. 6), (*ii*) the web extract of a virgin adult female (1 web equivalent) or that of a sub-adult

female (2 web equivalents), and (*iii*) the web extract of a sub-adult female (1 web equivalent) or that of a sub-adult male (1 web equivalent).

2.3.4 Statistical analyses

Data were analysed with IBM SPSS 23 (UNICOM Systems, Inc., CA, USA). Because data were not normally distributed, they were analyzed with a Mann-Whitney U test. Data analyses included the proportion of time males spent on each test stimulus relative to the total bioassay time, and the proportion of time males spent displaying courtship behaviour relative to the time they spent on a test stimulus.

2.4 Results

2.4.1 Web experiments

Bioassay males spent twice as much time on frames bearing the web of a virgin adult female than on empty control frames ($U = 196$, $N_1 = N_2 = 25$, $p = 0.021$), and courted exclusively on the web-bearing frames ($U = 87.5$, $N_1 = N_2 = 25$, $p < 0.001$; Fig. 2.1, Exp. 1). Males spent twice as much time, and displayed courtship behaviour longer, on frames bearing the web of a virgin adult female than on frames bearing the web of a sub-adult female (proportion of time spent: $U = 457$, $N_1 = N_2 = 25$, $p = 0.001$; proportion of time displaying courtship: $U = 562.5$, $N_1 = N_2 = 25$, $p < 0.001$; Fig. 2.1, Exp. 2). Conversely, males spent as much time on frames bearing the web of a sub-adult female or no web ($U = 268$, $N_1 = N_2 = 25$, $p = 0.376$; Fig. 2.1, Exp. 3), with only two of 25 males displaying courtship on web-bearing frames ($U = 337.5$, $N_1 = N_2 = 25$, $p = 0.153$; Fig. 2.1, Exp. 3). However, males spent twice as much time on frames bearing the web of a sub-adult female than on frames bearing the web of a sub-adult male, courting on neither type of frame (proportion of time spent: $U = 142.5$, $N_1 = N_2 = 25$, $p = 0.001$; proportion of time displaying courtship: $U = 312.5$, $N_1 = N_2 = 25$, $p = 1.000$; Fig. 2.1, Exp. 4). Males spent as much time on frames bearing the web of a sub-adult male or no web and courted on neither type of frame (proportion of time spent: $U = 220$, $N_1 = N_2 = 25$, $p = 0.072$; proportion of time courting: $U = 312.5$, $N_1 = N_2 = 25$, $p = 1.000$; Fig. 2.1, Exp. 5).

2.4.2 Web extract experiments

Bioassay males spent 4.5 times longer on filter paper treated with web extract of a virgin adult female (1 web equivalent) than on filter paper treated with web extract of a sub-adult female (1 web equivalent) ($U = 45.5$, $N_1 = N_2 = 25$, $p < 0.001$; Fig. 2.1, Exp. 6), courting exclusively on the former test stimulus ($U = 87.5$, $N_1 = N_2 = 25$, $p < 0.001$; Fig. 2.1, Exp. 6). Similarly, males spent 2.5 times longer on filter paper treated with web extract of a virgin adult female (1 web equivalent) than on filter paper treated with web extract of a sub-adult female (2 web equivalents) ($U = 106$, $N_1 = N_2 = 25$, $p < 0.001$; Fig. 2.1, Exp. 7), again courting exclusively on the former test stimulus ($U = 75$, $N_1 = N_2 = 25$, $p < 0.001$; Fig. 2.1, Exp. 7). Conversely, males spent as much time on filter paper treated with web extract of a sub-adult female or sub-adult male ($U = 285.5$, $N_1 = N_2 = 25$, $p = 0.600$; Fig. 2.1, Exp. 8), courting on neither test stimulus ($U = 312.5$, $N_1 = N_2 = 25$, $p = 1.000$; Fig. 2.1, Exp. 8).

2.5 Discussion

Our data support the hypothesis that adult female *S. grossa* are semiochemically cryptic to mate-seeking males. Males spent as much time on frames bearing the web of a sub-adult female as they did on control frames bearing no web (Fig. 2.1, Exp. 3). Furthermore, only two (5%) of 39 males that made physical contact with webs of sub-adult females (Exps. 2-4) displayed courtship behaviour, suggesting that webs of sub-adult females lack the physical or semiochemical cues that signal the presence of a receptive female to males. In contrast, the webs of virgin adult females both significantly retained males and prompted male courtship behaviour compared to concurrently presented empty control frames (Exp. 1) or webs of sub-adult females (Exp. 2). These data provide evidence that webs of adult virgin females, but not sub-adult females, carry sex pheromone that mate-seeking males detect.

Alternatively, webs of sub-adult females may simply carry less pheromone than those of adult females and thus be much harder to detect by males. This alternative explanation was inspired by two considerations: (1) webs of adult females have twice as

much silk and thus may carry twice as much pheromone than webs of sub-adult females; and (2) adult males spent significantly more time on webs of sub-adult females than on webs of sub-adult males (Exp. 4), suggesting that adult males might still be able to “read” physical or semiochemical web cues that reveal the presence of a sub-adult female.

To address whether physical or semiochemical web cues reveal the presence of a sub-adult female instead of a sub-adult male, we tested web extracts, thus excluding any effects of sex-specific silk or web architecture on the responses of males. Testing web extracts, instead of webs, also allowed us to compensate for possibly disparate amounts of pheromone associated with the 2-fold mass differential between webs of adult and sub-adult females. Yet, compared to 1-web-extract equivalent of virgin adult females, both 1- and 2-web-extract equivalents of sub-adult females failed to retain males and to prompt male courtship behaviour (Exps. 6, 7), indicating that the sex pheromone is either absent from webs of sub-adult females or far below detection threshold for most males. This conclusion is supported by evidence that web extracts of sub-adult females and sub-adult males were equally ineffective in retaining males or inducing male courtship (Exp. 8), and that no one single male-initiated courtship on filter paper treated with web extract of sub-adult females (Exps. 6-8). These results imply that the preference of adult males for webs of sub-adult females over those of sub-adult males (Exp. 4) is likely due to differential physical web cues (web architecture, silk-microstructure) rather than differential semiochemicals. The discriminant web characteristic(s) of sub-adult females and sub-adult males, however, remain(s) unknown as web architecture only of adult theridiids has been studied thus far (Benjamin & Zschokke, 2003). When male *S. ocreata* and *S. malitiosa* discriminated between the silk of adult virgin females and sub-adult females (Baruffaldi & Costa, 2010; Roberts & Uetz, 2005), they may have sensed differences either in silk micro-structure or pheromone presence.

Even though web architecture or silk micro-structure may allow *S. grossa* males to discriminate between webs of sub-adult females (prospective mates) and sub-adult males, this information is likely effective only upon physical contact with webs. Cobweb-building spiders generally have poor vision (Foelix, 2015; Uhl & Elias, 2011) and thus would not be able to discriminate between webs based on their visual characteristics. It

follows that sub-adult females lack any long-range apparency to mate-seeking males. Being cryptic to males is adaptive to sub-adult females that are in sexual conflict with adult males cohabiting their webs. Cohabiting males kleptoparasitize and curtail the female's opportunity of prey capture (Erez et al., 2005; Segev et al., 2003) and mate cannibalism (Biaggio et al., 2016; Uhl et al., 2015).

In conclusion, our data demonstrate apparent semiochemical crypsis of sub-adult *S. grossa* females to mate-seeking adult males. This crypsis is likely adaptive to females that are in sexual conflict with adult males cohabiting their webs.

2.6 Acknowledgments

We thank Paul J Watson and one anonymous reviewer for constructive comments on the manuscript. The research was supported by a Graduate Fellowship from Simon Fraser University to AF and by a Natural Sciences and Engineering Research Council of Canada (NSERC) – Industrial Research Chair to GG, with Scotts Canada Ltd. as the industrial sponsor.

2.7 References

Anava, A., & Lubin, Y. (1993). Presence of gender cues in the web of a widow spider, *Latrodectus revivensis*, and a descrition of courtship behaviour. *Bull.Br.Arachnol.Soc.*, *9*, 119–122.

Andrade, M. C. B. (1996). Sexual selection for male sacrifice in the Australian Redback Spider. *Science*, *271*, 70–72. http://doi.org/10.1126/science.271.5245.70

Arnqvist, G., & Nilsson, T. (2000). The evolution of polyandry: Multiple mating and female fitness in insects. *Animal Behaviour*, *60*, 145–164. http://doi.org/10.1006/anbe.2000.1446

Baruffaldi, L., & Costa, F. G. (2010). Changes in male sexual responses from silk cues of females at different reproductive states in the wolf spider *Schizocosa malitiosa*. *Journal of Ethology*, *28*, 75–85. http://doi.org/10.1007/s10164-009-0158-8

Baruffaldi, L., Costa, F. G., Rodríguez, A., & González, A. (2010). Chemical communication in *Schizocosa malitiosa*: Evidence of a female contact sex pheromone and persistence in the field. *Journal of Chemical Ecology*, *36*, 759–767. http://doi.org/10.1007/s10886-010-9819-x

Bel-Venner, M. C., & Venner, S. (2006). Mate-guarding strategies and male competitive ability in an orb-weaving spider: results from a field study. *Animal Behaviour*, *71*, 1315–1322. http://doi.org/10.1016/j.anbehav.2005.08.010

Bellmann, H. (2010). *Der Kosmos-Spinnenführer* (1. Edition). Stuttgart, Germany: Kosmos.
Benjamin, S. P., & Zschokke, S. (2003). Webs of theridiid spiders: construction, structure and evolution. *Biological Journal of the Linnean Society*, *78*, 293–305. http://doi.org/10.1046/j.1095-8312.2003.00110.x
Biaggio, M. D., Sandomirsky, I., Lubin, Y., Harari, A. R., & Andrade, M. C. B. (2016). Copulation with immature females increases male fitness in cannibalistic widow spiders. *Biology Letters*, *12*, 1–5.
Braun, R. (1956). Zur Biologie von *Teutana triangulosa*. *Zeitschrift für Wissenschaftliche Zoologie*, *159*, 255–318.
Buskirk, R., Frohlich, C., & Ross, K. (1984). The natural selection of sexual cannibalism. *The American Naturalist*, *123*, 612–625. http://doi.org/10.1086/284227
Chapman, T., Arnqvist, G., Bangham, J., & Rowe, L. (2003). Sexual conflict. *Trends in Ecology & Evolution*, *18*, 41–47. http://doi.org/10.1016/S0169-5347(02)00004-6
Cory, A.-L., & Schneider, J. M. (2017). Effects of social information on life history and mating tactics of males in the orb-web spider *Argiope bruennichi*. *Ecology and Evolution*, *8*, 344–355. http://doi.org/10.1002/ece3.3672
Dodson, G. N., & Beck, M. W. (1993). Pre-copulatory guarding of penultimate females by male crab spiders, *Misumenoides formosipes*. *Animal Behaviour*, *46*, 951–959. http://doi.org/10.1006/anbe.1993.1276
Eberhard, Guzman-Gomez, S., & Catley, K. M. (1993). Correlation between spermathecal morphology and mating systems in spiders. *Biological Journal of the Linnean Society*, *50*, 197–209. http://doi.org/10.1111/j.1095-8312.1993.tb00926.x
Eberle, M., & Kappeler, P. M. (2004). Selected polyandry: Female choice and inter-sexual conflict in a small nocturnal solitary primate (*Microcebus murinus*). *Behavioral Ecology and Sociobiology*, *57*, 91–100. http://doi.org/10.1007/s00265-004-0823-4
Engel, K. C., Schweizer, R., Vogel, H., Ayasse, M., Ruther, J., Stockl, J., & Steiger, S. (2016). A hormone-related female anti-aphrodisiac signals temporary infertility and causes sexual abstinence to synchronize parental care. *Nature Communications*, *7*, 11035. http://doi.org/10.1038/ncomms11035
Erez, T., Schneider, J. M., & Lubin, Y. (2005). Is male cohabitation costly for females of the spider *Stegodyphus lineatus* (Eresidae)? *Ethology*, *111*, 693–704. http://doi.org/10.1111/j.1439-0310.2005.01090.x
Estrada, C., Yildizhan, S., Schulz, S., & Gilbert, L. E. (2010). Sex-specific chemical cues from immatures facilitate the evolution of mate guarding in *Heliconius* butterflies. *Proceedings of the Royal Society B: Biological Sciences*, *277*, 407–413. http://doi.org/10.1098/rspb.2009.1476
Fahey, B. F., & Elgar, M. A. (1997). Sexual cohabitation as mate-guarding in the leaf-curling spider *Phonognatha graeffei* Keyerling (Araneoidae, Araneae). *Behavioral Ecology and Sociobiology*, *40*, 127–133.
Foelix, R. (2015). *Biologie der Spinnen* (3rd ed.). Frankfurt am Main: Chimaira.
Garb, J. E., & Hayashi, C. Y. (2013). Molecular evolution of alpha-latrotoxin, the exceptionally potent vertebrate neurotoxin in black widow spider venom. *Molecular Biology and Evolution*, *30*, 999–1014.

http://doi.org/10.1093/molbev/mst011
Gavrilets, S. (2000). Rapid evolution of reproductive barriers driven by sexual conflict. *Nature*, *403*, 886–889. http://doi.org/10.1038/35002564
Gerhardt, U. (1924). Neue Sexualbiologische Spinnenstudien. *Zoomorphology*, 3, 567–618.
Gwinner-Hanke, H. (1970). Zum Verhalten zweier stridulierender Spinnen *Steatoda bipunctata* Linne und *Teutana grossa* Koch (Theridiidae, Araneae), unter besonderer Berücksichtigung des Fortpflanzungsverhaltens. *Ethology*, *27*, 649–678. http://doi.org/10.1111/j.1439-0310.1970.tb01893.x
Jackson, R. R. (1986). Cohabitation of males and juvenile females: a prevalent mating tactic of spiders. *Journal of Natural History*, *20*, 1193–1210. http://doi.org/10.1080/00222938600770791
Kasumovic, M. M., & Andrade, M. C. B. (2006). Male development tracks rapidly shifting sexual versus natural selection pressures. *Current Biology*, *16*, 242–243. http://doi.org/10.1016/j.cub.2006.03.017
Knoflach, B. Z. (2004). Diversity in the copulatory behaviour of comb-footed spiders (Araneae, Theridiidae). *Denisia*, *12*, 161–256.
Miller, G. L., & Miller, P. R. (1986). Pre-courtship cohabitation of mature male and penultimate female *Geolycosa turricola*. *Journal of Arachnology*, *14*, 133–134.
Miyashita, T., & Hayashi, H. (1996). Volatile chemical cue elicits mating behavior of cohabiting males of *Nephila clavata*. *Journal of Arachnology*, *24*, 9–15.
Roberts, J. A., & Uetz, G. W. (2005). Information content of female chemical signals in the wolf spider, *Schizocosa ocreata*: male discrimination of reproductive state and receptivity. *Animal Behaviour*, *70*, 217–223. http://doi.org/10.1016/j.anbehav.2004.09.026
Schulz, S. (2013). Spider pheromones - a structural perspective. *Journal of Chemical Ecology*, *39*, 1–14. http://doi.org/10.1007/s10886-012-0231-6
Scott, C., Gerak, C., McCann, S., & Gries, G. (2018). The role of silk in courtship and chemical communication of the false widow spider, *Steatoda grossa* (Araneae: Theridiidae). *Journal of Ethology*, *36*, 191–197. http://doi.org/10.1007/s10164-017-0539-3
Segev, O., Ziv, M., & Lubin, Y. (2003). The male mating system in a desert widow spider. *Journal of Arachnology*, *31*, 379–393. http://doi.org/10.1636/s01-101
Symonds, M. R. E., & Elgar, M. A. (2008). The evolution of pheromone diversity. *Trends in Ecology and Evolution*, *23*, 220–228. http://doi.org/10.1016/j.tree.2007.11.009
Uhl, G. (2013). Spider olfaction: attracting, detecting, luring and avoiding. In *Ecophysiology* (pp. 141–157). New York, NY: Springer. http://doi.org/10.1007/978-3-642-33989-9
Uhl, G., & Elias, D. (2011). Communication. In *Spider Behaviour* (pp. 127–189). Cambridge: Cambridge University Press.
Uhl, G., Zimmer, S. M., Renner, D., & Schneider, J. M. (2015). Exploiting a moment of weakness: male spiders escape sexual cannibalism by copulating with moulting females. *Scientific Reports*, *5*, 16928. http://doi.org/10.1038/srep16928
Watson, P. J. (1986). Transmission of a female sex pheromone thwarted by males in the spider *Linyphia litigiosa* (Linyphiidae). *Science*, *233*(July), 219–221.

Watson, P. J. (1990). Female-enhanced male competition determines the first mate and principal sire in the spider *Linyphia litigiosa* (Linyphiidae). *Behavioral Ecology and Sociobiology*, *26*, 77–90. http://doi.org/10.1007/BF00171577

Watson, P. J. (1991). Multiple paternity and first mate sperm precedence in the Sierra Dome Spider, <i>Linyphia litigiosa Keyserling (Linyphiidae). *Animal Behaviour*, *41*, 135–148.

Wise, D. H. (2006). Cannibalism, food limitation, intraspecific competition, and the regulation of spider populations. *Annual Review of Entomology*, *51*, 441–465. http://doi.org/10.1146/annurev.ento.51.110104.150947

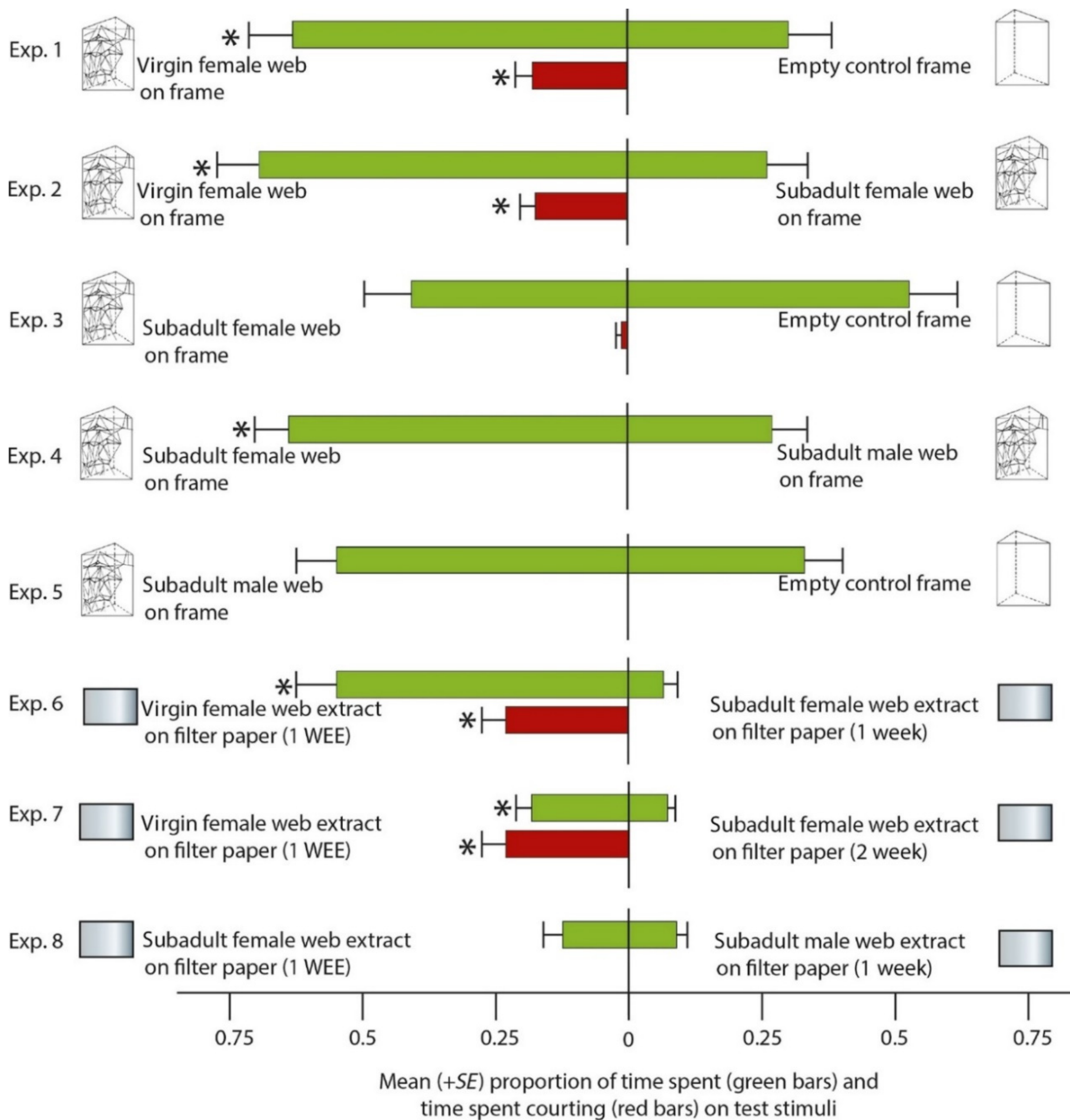


Figure 2.1: Bioassay results. Mean (+ SE) proportion of time that male *Steatoda grossa* (*i*) stayed on a test stimulus relative to the total bioassay time (green bars), and (*ii*) displayed courtship on a test stimulus relative to the time spent on it (red bars). In each of experiments 1-8 (N = 25 each), an asterisk (*) indicates a statistically significant behavioural response to a test stimulus (Mann-Whitney U test, $p < 0.05$). Note: (*i*) 1 WEE = 1 Web Extract Equivalent; (*ii*) lack of a red bar indicates no occurrence of courtship behaviour; (*iii*) males were not re-tested within experiments.

Chapter 3: Origin, structure and functional transition of sex pheromone components in a false widow spider[1]

[1]The corresponding manuscript has been published in *Communications Biology* (Volume 5, 1156, 2022), with the following authors: Andreas Fischer, Regine Gries, Santosh K. Alamsetti, Emmanuel Hung, Andrea C. R. Torres, Sula Fernando, Sanam Meraj, Weiwu Ren, Robert Britton, Gerhard Gries

3.1 Abstract

Female web-building spiders disseminate pheromone from their webs that attracts mate-seeking males and deposit contact pheromone on their webs that induces courtship by males upon arrival. The source of contact and mate attractant pheromone components, and the potential ability of females to adjust their web's attractiveness, have remained elusive. Here, we report three new contact pheromone components produced by female false black widow spiders, *Steatoda grossa*: *N*-4-methylvaleroyl-*O*-butyroyl-L-serine, *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine and *N*-4-methylvaleroyl-*O*-hexanoyl-L-serine. The compounds originate from the posterior aggregate silk gland, induce courtship by males, and web pH-dependently hydrolyse at the carboxylic-ester bond, giving rise to three corresponding carboxylic acids that attract males. A carboxyl ester hydrolase (CEH) is present on webs and likely mediates the functional transition of contact sex pheromone components to the carboxylic acid mate attractant pheromone components. As CEH activity is pH-dependent, and female spiders can manipulate their silk's pH, they might also actively adjust their webs' attractiveness.

3.2 Introduction

Attracting or finding a mate is essential for all sexually reproductive animal species[1–3]. The process is mediated by long-range communication signals that have chemical, auditory, visual, vibratory or multimodal characteristics[4–7]. Chemicals such as pheromones are deemed the oldest form of (sexual) communication signals[8] and have evolved in various animal taxa including mammals[9], myriapods[10], crustaceans[11], and insects[12–15]. Airborne pheromones have signal functions in the context of aggregation[16], territorial marking[17], warning[18], nest defence[19], and reproduction[20,21]. Volatile sex pheromones attract prospective mates[7], whereas cuticle-bound mate recognition pheromones impart reproductive isolation and insect speciation[22].

Sex pheromones have been almost extensively studied in insects[12–15]. Beetles, moths, ants and wasps all produce, and release, pheromones from specific glands located in various parts of their body[23]. Many insects can actively time their pheromone production and release, and modulate the amount of pheromone they emit[7,14,24]. Pheromones are perceived by olfactory receptors on the insects' antennae[12] involving complex molecular interactions between pheromone receptors and their pheromone ligands[25–27]. More than 3000 insect pheromones have already been identified[13]. Using the insects' antennae as an analytical tool to help locate candidate pheromone components in complex analytical samples[28] has been instrumental for identifying many of these pheromones, particularly those that occur at trace quantities[29]. In contrast, to date only 12 spider sex pheromones have been identified and neither their site of production nor their site of reception is known[30,31].

Web-building spiders are multi-modal communicators, using primarily pheromonal and vibratory communication signals[32]. Pheromones play major roles during habitat selection[30,33,34], mate competition[35,36], courtship[37], and mate choice[33,38]. Unlike insects that typically disseminate pheromones from specific gland tissues[7,24], female spiders deposit pheromones on their silken webs[39]. Their webs attract males over long distances[40] and upon contact elicit courtship in males[41], implying the release of mate attractant pheromone components from the web and the presence of contact pheromone

components on the web[39]. To date, it is not known whether (*i*) spider pheromones originate from a silk gland, (*ii*) mate attractant and contact pheromone components are structurally and functionally related, and (*iii*) female spiders can actively modulate the release of mate attractant pheromone components from their webs.

Pheromone components that female spiders deposit on their webs and that induce courtship by males upon contact have been identified in the linyphiid spider *Linyphia triangularis*[42] and the widow spiders *Latrodectus hasselti*[43] and *L. hesperus*[44]. Female *L. triangularis* deposit (*R*)-3-[(*R*)-3-hydroxybutyryloxy]-butyric acid (**1**) on their webs, whereas female *L. hasselti* and *L. hesperus* deposit serine derivatives [*N*-3-methyl-butyryl-*O*-(S)-2-methylbutyryl-*L*-serine methyl ester[43] (**2**); *N*-3-methylbutanoyl-*O*-methylpropanoyl-*L*-serine methyl ester[44] (**3**)(Fig. 3.1a)]. Both **1** and its breakdown monomer, (*R*)-3-hydroxybutyric acid (**4**), induce courtship by male *L. triangularis*[31,42]. These results imply that the breakdown of contact pheromone components could engender more volatile pheromone components that then attract males. We predicted that a potential breakdown of *Latrodectus* serine methyl esters could be catalysed by a carboxyl ester-hydrolase, which was found on *L. hesperus* webs[45]. As enzyme activity is pH-dependent[46], and spider females might be able to adjust their silk's pH[47], we surmised that *Latrodectus* females possibly modulate the breakdown dynamics of their serine methyl ester deposits, and thus the release of their mate attractant pheromone components.

Here we worked with the globally invasive and synanthropic false black widow spider, *Steatoda grossa* (Theridiidae, Araneae)[48]. *Steatoda grossa* inhabits predominantly buildings, where it reproduces year-round irrespective of season[48,49]. As *Steatoda* and *Latrodectus* spiders are close phylogenetic relatives[50–52], we anticipated that *S. grossa* would produce pheromone components structurally resembling those of *Latrodectus*. We report the identification of *S. grossa* contact pheromone components, their origin, and breakdown to volatile mate attractant pheromone components, likely catalysed by a pH-dependent carboxyl ester-hydrolase present on the females' webs.

3.3 Results and Discussion

3.3.1 Identification of contact pheromone components

To obtain analyte for the identification of contact pheromone components, we allowed 93 sexually mature adult virgin females and – for comparative analysis – 70 sexually immature subadult females[37] three days to build their webs on a prism scaffold (Fig. 3.1b), building upon previous results that only mature females produce pheromone components[37]. We then methanol-extracted pooled webs from each of the two female groups[37] and analysed extracts by gas chromatography-mass spectrometry (GC-MS). These analyses revealed seven compounds (**5**–**11** in Fig. 3.1d; pyrrolidin-2-one (**5**), 4-hydroxyhydrofuran-2(3*H*)-one (**6**), nonanoic acid (**7**), dodecanoic acid (**8**), 6-methylheptanamide (**9**), octanamide (**10**), 4,6-dimethyl heptanamide (**11**), that were unique to sexually mature females. To test compounds **5**–**11** for their ability to induce courtship by male spiders, we treated one piece of filter paper on a T-rod apparatus (Fig. 3.1c) with a synthetic blend of **5**–**11** (Exp. 1), or with web extract (positive control; Exp. 2), and the corresponding filter paper with a solvent control. As only web extract, but not the blend of **5**–**11**, elicited courtship by males ($N_1 = N_2 = 20$, W = 370, $P < 0.001$, Exp. 1+2, Fig. 3.1e;), it follows that **5**–**11** are not contact pheromone components. Concerned that the contact pheromone components were too polar or too large to chromatograph in GC-MS analyses, we fractionated web extract by high performance liquid chromatography (HPLC) and bioassayed each of 20 HPLC fractions for courtship responses by males on the T-rod apparatus. All fractions that elicited courtship behaviour by males (Appendix Fig. 3.1) were then analysed by HPLC-tandem mass spectrometry (MS/MS) and by nuclear magnetic resonance (^{1}H NMR) spectroscopy. HLPC-MS/MS analyses revealed an unknown compound (**12**) with a molecular formula of $C_{13}H_{23}NO_5$ and fragmentation ions 186, 274 (M+1) and 296 (M+Na), indicating a molecular weight of 273 (Fig. 3.2a). Both the molecular formula and the molecule's weight matched those of the serine methyl ester (**2**) in web extracts of *L. hesperus* (Fig. 3.1a). Yet, the ^{1}H NMR spectrum of unknown **12** (Appendix Fig. 3.2) did not support an ester functionality, and GC-MS analyses of *S. grossa* web extracts did not provide any evidence for the presence of a serine methyl ester. Predicting then that **12** was an acid (rather than an ester) which –

due to its polar nature – would not chromatograph well in GC-MS analyses, we esterified crude web extract with trimethylsilyldiazomethane[53] and reanalysed aliquots of this extract by GC-MS. These analyses revealed not only one, but three serine methyl ester derivatives (Fig. 3.2b; *N*-4-methylvaleroyl-*O*-butyroyl-*L*-serine methyl ester (**13**), *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine methyl ester (**14**), and *N*-4-methylvaleroyl-*O*-hexanoyl-*L*-serine methyl ester (**15**)), supporting our prediction that female *S. grossa* produce serine derivatives with a carboxyl (acid) rather than a methyl ester functionality. To infer the structure of the unknown acid **12**, we drew on evidence that its 186 mass fragment (Fig. 3.2a) was also present in serine methyl esters **2** and **3** produced by *L. hasselti* and *L. hesperus* (Fig. 3.1a). For the 186 mass fragment of **12**, this meant that the acyl bound to the nitrogen atom had six carbon atoms, instead of five (as in esters **1** and **2**), with 4 possible isomers: 2-, 3- or 4-methylpentanoyl and hexanoyl. For the molecular ion of **12** to be *m/z* 173, the second acyl bound to the oxygen atom had to have only four carbon atoms with either butyryl or isobutyryl configuration. Of eight possible synthetic isomers (see SI), only *N*-4-methylpentanoyl-*O*-butyryl-L-serine (**12**, Fig. 3.2a) had HPLC-MS/MS spectrometric and retention characteristics entirely consistent with *S. grossa* produced **12**. Moreover, the corresponding synthetic methyl ester of **12,** *N*-4-methylpentanoyl-*O*-butyryl-*L*-serine methyl ester, had retention and mass spectral characteristics entirely consistent with those of the most abundant serine methyl ester **13** in esterified web extracts of *S. grossa* (Fig. 3.2b).

All three serine methyl ester derivatives had similar mass spectra (Fig. 3.2c), indicating a conserved molecular structure with differences only in the acyl groups of the molecules. Ester **13** [retention index (RI): 1843] and ester **14** (RI: 1890) had identical mass spectra (Fig. 3.2c) but their RI differential of 43 units indicated a methyl branch in **13**. The RI of ester **15** (2074) was about 200 RI units higher than that of ester **14** implying the presence of a higher homologue with two additional carbon atoms. To assign definitive molecular structures to esters **13** and **15**, we synthesized multiple standards (see Appendix: Syntheses). Of these, *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine methyl ester and *N*-4-methylvaleroyl-*O*-hexanoyl-L-serine methyl ester had mass spectrometric and retention characteristics entirely consistent with those of the serine methyl ester derivatives **13** and **15**, respectively, in esterified web extracts. Moreover, in

HPLC-MS/MS analyses, the corresponding synthetic acids (*N*-4-methylvaleroyl-*O*-isobutyroyl-*L*-serine (**12**); *N*-4-methylvaleroyl-*O*-isobutyroyl-*L*-serine (**16**); *N*-4-methylvaleroyl-*O*-hexanoyl-*L*-serine (**17**); Fig. 3.2a) had retention times and mass spectra entirely consistent with those produced by female *S. grossa* and present in web extract. In T-rod (Fig. 3.1c) bioassays, a ternary blend of the synthetic acids **12**, **16** and **17**, tested at one web equivalent, elicited courtship behaviour by *S. grossa* males comparable to web extract (Exp. 3 *vs.* Exp. 4: $N_1 = N_2 = 20$, Z = -0.39, $P = 0.521$, Fig. 3.2d), indicating that all essential contact pheromone components were present in this synthetic blend. The seven volatile components **5–11** unique to sexually mature females (Fig. 3.1d) did not enhance the behavioural activity of the ternary acid blend (**12**, **16, 17**) (Exp 4. *vs.* Exp. 5: $N_1 = N_2 = 20$, Z = 0.03, $P = 0.488$, Fig. 3.2d) nor did they induce any courtship behaviour on their own (Exp. 6, Fig. 3.2d). In contrast, the ternary acid blend induced courtship behaviour in a dose-dependent manner (Exps. 7–11: $\chi^2 = 61.75$, df = 4, $P < 0.001$; Appendix Fig. 3.3). Binary blends of the acids also induced courtship behavior, but their effect differed according to blend constituents (Exps. 12–15: $\chi^2 = 11.19$, df = 3, $P = 0.010$; Appendix Fig. 3.4). Acids **12** and **16** tested singly elicited courtship as effectively as in binary combination (Exps. 16–18: $\chi^2 = 3.65$, df = 2, $P = 0.160$; Appendix Fig. 3.5).

3.3.2 Origin of contact pheromone components

Silk glands have been hypothesized[54], but never been experimentally shown, to produce sex pheromones. Moreover, the specific silk gland (out of eight possible glands) that produces the pheromone components has never been determined. With the *S. grossa* contact pheromone components now identified and key spectrometric data of the most abundant component (**12**) in hand (Fig. 3.2a), we proceeded to trace its origin. For all analyses, we cold-euthanized spiders, extracted body tissue in a methanol/saline solution[55], centrifuged extracts, and analysed aliquots of each tagma or tissue sample by HPLC-MS for the quantity of **12** and **16**. Because contact pheromone components **12** and **16** coeluted in these analyses, we quantified their combined amount. As only the abdomen, but not the cephalothorax, of spiders contained **12** & **16** (Exp. 19: N = 22, W = 21, $P = 0.004$, Fig. 3.3a), we then screened abdominal hemolymph and five specific abdominal tissues, including all eight silk glands combined, for the presence of **12** & **16**.

With only silk gland samples containing **12** & **16** (Exp. 20: N = 20, χ^2 = 70.96, df = 6, P < 0.001, Fig. 3.3b), we analysed glands separately and found that it was the posterior aggregate gland that exclusively, or most abundantly, contained **12** & **16** (Exp. 21: N = 30, χ^2 = 36.00, df = 6, P < 0.001, Fig. 3.3c). Although not specifically tested, it is likely that the posterior aggregate gland also produces contact pheromone component **17**.

3.3.3 Transition of contact pheromone components to volatile mate attractant pheromone components

Long-distance orientation of male spiders to mate attractant pheromone components emanating from female *S. grossa* webs was tested in Y-tube, moving-air olfactometers[56], using web extract (instead of webs) as the test stimulus (Fig. 3.4a). When offered a choice between web extract and a solvent control, males were attracted to web extract (Exp. 22: N = 21, P = 0.013, Fig. 3.4b). However, when offered a choice between the blend of volatile compounds **5–11** unique to sexually mature females (Fig. 3.1d) and a solvent control, males exhibited no attraction responses (Exp. 23: N = 20, P = 0.588, Fig. 3.4b). These data implied that the mate attractant pheromone components were not readily detectable and possibly arose from chemical reactions occurring on the web. Drawing on a previous report[31,42] that the dimer contact pheromone **1** of the spider *L. triangularis* breaks down to a volatile monomer attractant (**4**) (Fig. 3.1a), we hypothesized (Fig. 3.4c) that the contact pheromone components **12**, **16** and **17** of female *S. grossa* hydrolyse over time at the carboxylic-ester bond, giving rise to the amide *N*-4-methylvaleroyl-*L*-serine (**18**) and three corresponding carboxylic acids [butyric (**19**), isobutyric (**20**), hexanoic (**21**)], and that these volatile acids then attract males. Realizing the difficulty to quantify the release of these acids over time, we instead quantified the accumulating amide **18** as a proxy for the breakdown of contact pheromone components (Fig. 3.4c). Our breakdown hypothesis was supported by data showing a significantly higher breakdown ratio [**18** / (**18** + **12** + **16** + **17**)] in extracts of 14-day-old webs than in those of freshly spun (0-day-old) webs (Exp. 24: W= 637, $N_{0\ days} = N_{14\ days}$ = 70, p < 0.001, Fig. 3.4d). Moreover, our attraction hypothesis was supported by Y-tube olfactometer data (Fig. 3.4e) showing that males are attracted to a blend of the four breakdown products **18–21** (Exp. 25: N = 29, P = 0.030) and to a blend of the three

carboxylic acids **19**–**21** (Exp. 26: N = 26, $P = 0.006$), but not to the amide **18** (Exp. 27: N = 25, $P = 0.500$). Tested on its own, amide **18** also did not elicit any courtship behaviour by males, nor did it increase the activity of the contact pheromone components **12**, **16** and **17** which – when tested as a ternary blend in parallel – effectively induced courtship (Exp. 29–31: $\chi^2 = 12.78$, df = 2, $P < 0.001$, Appendix Fig. 3.6).

To substantiate our conclusion that the carboxylic acids **19**–**21** function as mate attractant pheromone components of female *S. grossa*, we formulated these acids in mineral oil[9] and tested them as a trap lure in building hallways with low *S. grossa* infestations. Over the course of 16 weeks, carboxylic acid-baited traps captured nine *S. grossa* males, whereas corresponding control traps captured only 1 male, confirming the mate attractant pheromone function of the carboxylic acids (Exp. 28: N = 10, $P = 0.011$, Fig. 3.4f).

3.3.4 Mechanisms underlying the transition of contact pheromone components to mate attractant pheromone components.

We hypothesized that the chemical breakdown of the contact pheromone components **12**, **16** and **17**, and the release of the carboxylic acids **19, 20** and **21** as mate attractant pheromone components are catalysed by one or both of two non-mutually exclusive mechanisms: (1) the activity of a web-borne carboxyl ester-hydrolase (CEH) and (2) direct saponification of the contact pheromone components. Both mechanisms are pH-dependent. To test our prediction that breakdown rates of contact pheromone components are positively correlated with the webs' pH, we allowed each of 70 spiders to spin two webs. We used one web from each spider to quantify the contact pheromone components (**12**, **16** and **17**) and their amide breakdown product (**18**), and the other web to determine its pH. For pH measurements, we determined the pH of each web by adding the web to a small volume of water which served as a conductor for the pH-meter[57]. Plotting the data revealed a significant positive correlation between the pH of webs and the chemical breakdown rate (ratio of **18** / (**12 + 16** + **17 + 18**)) (Exp. 32: $F_{1,69} = 108.44$, $p < 0.001$; Fig. 3.5a).

To further determine whether pH directly affects the hydrolysis of contact pheromone components, we exposed synthetic **12** to pH 7 or pH 4 buffer solutions, and to aprotic acetonitrile. While both buffer solutions afforded significantly greater breakdown rates than the aprotic control solution (Exp. 33–35, $\chi^2 = 25.84$, df = 2, $p < 0.001$, Fig. 3.5b), the effect size was 10-fold lower than that measured on webs (Exp. 24). Thus, the pH as a direct (single) factor is insufficient to catalyse the hydrolysis of contact pheromone components (**12, 16, 17**) to mate attractant pheromone components (**19**–**21**). However, if there were a carboxyl ester hydrolase (CEH) to be present on *S. grossa* webs, as there is on *L. hesperus* webs[45], then pH could affect the enzymatic activity of a CEH, and thereby the hydrolysis of contact pheromone components. With *S. grossa* and *L. hesperus* being close phylogenetic relatives[51], we predicted that they produce not only similar serine-derived contact pheromone components[39] (see **3** and **12**) but also a similar or identical CEH to hydrolyse them. To test our prediction, we extracted webs in Sørensen buffer[58] from three groups of spiders: (1) adult virgin female *L. hesperus* (positive control, known to have a CEH[45]); (2) subadult sexually immature female *S. grossa* (deemed to have not yet produced a CEH) and (3) adult virgin sexually mature female *S. grossa* (predicted to have the same CEH as *L. hesperus*). To account for different amounts of silk produced by these three groups of spiders, we extracted five webs of *L. hesperus*, 20 webs of subadult *S. grossa*, and 10 webs of adult *S. grossa* in each of three replicates and submitted extracts for comparative proteomics (CEH analyses) (see SI for detailed methods). The CEH was present in all three samples of *L. hesperus* and adult *S. grossa* and – surprisingly – also in two of three samples of subadult *S. grossa*, possibly because some webs were produced by females about to become sexually mature. Conceivably, this CEH may – pH-dependently – hydrolyse the *S. grossa* contact pheromone components, with females manipulating enzyme activity by altering their webs' pH. Increasing their webs' pH would enhance the hydrolysis of contact pheromone components (Fig. 3.5b), and thus the release of mate attractant pheromone components, making their webs more attractive to mate-seeking males. This concept could be tested experimentally. Once engineered CEH becomes available, it could be placed on artificial (Halloween) spider web[30] with specific pH values and treated with synthetic contact pheromone component to measure hydrolysis rates.

Our study addresses significant questions about the communication ecology of web-building spiders. These unresolved questions were whether (1) spider pheromone originates from a silk gland, (2) mate attraction and courtship-inducing contact pheromone components are chemically interlinked, and (3) female spiders actively adjust pheromone dissemination from their web to attract males. Here, we provide definitive answers to questions 1 and 2, and we discuss data for question 3. First, we identified three previously unknown serine-derived contact pheromone components produced by *S. grossa* females: *N*-4-methylvaleroyl-*O*-isobutyroyl-*L*-serine (**12**); *N*-4-methylvaleroyl-*O*-isobutyroyl-*L*-serine (**16**); *N*-4-methylvaleroyl-*O*-hexanoyl-*L*-serine (**17**). We then show that these components originate from the posterior aggregate silk gland and – once web-borne – induce courtship by males. We further demonstrate a functional transition of these contact sex pheromone components to volatile mate attractant pheromone components. Web pH-dependently, the contact pheromone components hydrolyse at the carboxylic ester bond and give rise to three corresponding carboxylic acids that attract males. With increasing web pH (4–7), hydrolysis rates increase, and greater amounts of carboxylic acids (as hydrolysis products) are released. However, pH 7 alone is insufficient to induce biologically significant hydrolysis rates. Subjecting synthetic contact pheromone to a pH 7 buffer solution induced hydrolysis rates 10-fold lower than those measured on webs. These data imply that the hydrolysis is catalysed by an enzyme, most likely the carboxyl ester hydrolase that is present on *S. grossa* webs. This carboxyl ester hydrolase, pH-dependently, might hydrolyse the contact pheromone components, with the enzyme apparently being most active around pH 7. Our explanation of enzyme-catalysed contact pheromone hydrolysis is supported, in part, by pheromone studies of the widow spider *L. hesperus*, a phylogenetically close relative of *S. grossa*[50]. Female *L. hesperus* also produce a serine derivative contact pheromone component[44] (Fig. 3.1a) that is likely hydrolysed by a carboxyl ester hydrolase, reported to be present on *L. hesperus* webs[45].

Sustained dissemination of mate attractant pheromone components from a reservoir of web-borne contact pheromone components is adaptive for sessile web-building spiders. Sustained pheromone dissemination establishes a somewhat permanent information flow to potential signal recipients. This type of dissemination system is

reminiscent of pheromone dissemination from urine markings of murine rodents. Here, major urinary proteins bind to mate attractant pheromone components, and facilitate their slow release[59], thus prolonging the attractiveness of pheromonal markings[60].

If we accept the concept that in *S. grossa* an enzyme is involved in mediating the transition of contact pheromone components to mate attractant pheromone components, and if we apply the common knowledge that enzyme activity is pH dependent[46], and spiders lower the pH in their spinning apparatus to convert aqueous silk to solid silk threads[47,61,62], it follows that female *S. grossa* might be able to actively adjust their web's attractiveness to males. To date, only insects were known to actively time their pheromone production and dissemination[63], and to modulate the amount of pheromone they emit[64]. Our findings suggest, but do not prove, that web-building spiders might do this as well. With the pheromone system of *S. grossa* now known, potential manipulation by female spiders of their webs' pH, and thus their webs' attractiveness to mate-seeking males, can now be tested in the context of honest or dishonest signalling.

Our finding that the posterior aggregate silk gland is the source of contact pheromone components in *S. grossa* will help expedite pheromone identification in other spiders, provided – of course – that their pheromones originate from the same silk gland. Pheromone-producing glands often contain a sufficiently large amount of pheromone analyte for structural elucidation[7]. Many insect pheromones could be identified primarily because the pheromone-producing gland was known, and many glands could be extracted for pheromone accumulation and analysis[65,66]. For pheromone identification in web-building spiders, it would also be easier to extract and analyse the content of the pheromone-producing silk gland than to extract and analyse an entire web with, possibly, many more constituents.

3.4 Conclusions

In conclusion, our study reveals the intricate pheromonal communication system of *S. grossa,* as a model species for web-building spiders, and it provides incentive for comparative studies in other spider taxa.

3.5 Methods

3.5.1 Experimental spiders

Experimental spiders were maintained as previously reported[37]. Briefly, spiders were the F1 to F4 offspring of mated females collected from hallways of the Burnaby campus of Simon Fraser University (Burnaby, BC, CA). Upon hatching, juvenile spiders were housed individually in petri dishes (100 × 20 mm) and provisioned with the vinegar flies *Drosophila melanogaster*. Sub-adult spiders were fed with larvae of the mealworm beetle *Tenebrio molitor*. Each adult female spider was kept in a separate translucent 300-mL plastic cup (Western Family, CA) maintained at 22 °C under a reversed light cycle (12:12 h). Adult males and females were fed with black blow flies, *Phormia regina*. All spiders had access to water in cotton wicks. Water and food were provided once per week. Laboratory experiments were run during a reversed scotophase (0900 to 1700).

3.5.2 Identification of contact pheromone components: Preparation of web extracts (summer 2017; spring & summer 2018)

Each of 100 spiders was allowed to build her web for three days on a wooden triangular prism scaffold (30 × 25 × 22 cm)[44] of bamboo skewers (GoodCook, CA, USA) (Fig. 3.1b). After spiders were removed from the scaffold, their webs were reeled up with a glass rod (10 × 0.5 cm) and deposited in a 1.5-mL glass vial. Per web, 50 µL of methanol (99.9% HPLC grade, Fisher Chemical, ON, Canada) were added and the silk was extracted for 24 h at room temperature. Prior to analysis, the silk was removed, and the sample concentrated under a steady nitrogen stream to the desired concentration.

3.5.3 Identification of contact pheromone components: Analyses of web extracts by gas chromatography-mass spectrometry (GC-MS)

Aliquots (2 µL) of pooled and concentrated web extract (100 webs in 400 µL of solvent) were analysed by GC-MS, using a Varian Saturn Ion trap 2000 (Varian Inc., now Agilent Technologies Inc., Santa Clara, CA 95051, USA) and an Agilent 7890B GC coupled to a 5977A MSD, both fitted with a DB-5 GC-MS column (30 m × 0.25 mm ID, film thickness 0.25 µm). The injector port was set to 250 °C, the MS source to 230 °C,

and the MS quadrupole to 150 °C. Helium was used as a carrier gas at a flow rate of 35 cm s^{-1}, with the following temperature program: 50 °C held for 5 min, 10 °C min^{-1} to 280 °C (held for 10 min). Compounds were identified by comparing their mass spectra and retention indices (relative to aliphatic alkanes[67]) with those of authentic standards that were purchased or synthesized in our laboratory (Appendix Table 3.1).

3.5.4 Identification of contact pheromone components: High performance liquid chromatography (HPLC) of web extracts

Web extract of virgin adult female *S. grossa* was fractionated by high performance liquid chromatography (HPLC), using a Waters HPLC system (Waters Corporation, Milford, MA, USA; 600 Controller, 2487 Dual Absorbance Detector, Delta 600 pump) fitted with a Synergy Hydro Reverse Phase C_{18} column (250 mm × 4.6 mm, 4 µ; Phenomenex, Torrance, CA, USA). The column was eluted with a 1-mL/min flow of a solvent gradient, starting with 80% water (HPLC grade, EMD Millipore Corp., Burlington, MA, USA) and 20% acetonitrile (99.9% HPLC grade, Fisher Chemical, Ottawa, CA) and ending with acetonitrile after 10 min. A 60-web-equivalent extract was injected, and 20 1-min fractions were collected. Each HPLC fraction (containing 20 web-equivalents) was tested in T-rod bioassays (Fig. 3.1c) for the presence of contact pheromone components. All eight fractions that elicited courtship responses by males (Appendix Fig. 3.1) were analysed by HPLC-tandem MS/MS.

3.5.5 Identification of contact pheromone components: HPLC-tandem MS/MS of bioactive HPLC fractions

The bioactive HPLC fractions were analysed on a Bruker maXis Impact Quadrupole Time-of-Flight HPLC/MS System. The system consists of an Agilent 1200 HPLC fitted with a spursil C_{18} column (30 mm x 3.0 mm, 3 µ; Dikma Technologies, Foothill Ranch, CA, USA) and a Bruker maXis Impact Ultra-High Resolution tandem TOF (UHR-Qq-TOF) mass spectrometer. The LC-MS conditions were as follows: The mass spectrometer was set to positive electrospray ionisation (+ESI) with a gas temperature of 200 °C and a gas flow of 9 L/min. The nebulizer was set to 4 bar and the capillary voltage to 4200 V. The column was eluted with a 0.4-mL/min flow of a solvent

gradient, starting with 80% water and 20% acetonitrile and ending with 100% acetonitrile after 4 min. The solvent contained 0.1% formic acid to improve peak shape.

3.5.6 Identification of contact pheromone components: ^{1}H NMR analyses of a bioactive fraction

A single bioactive fraction (9–10 min) appeared in the HPLC-MS analysis to contain only a single compound. This fraction was then further investigated using ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum was recorded on a Bruker Advance 600 equipped with a QNP (600 MHz) using $CDCl_3$. Signal positions (δ) are given in parts per million from tetramethylsilane (δ 0) and were measured relative to the signal of the solvent (^{1}H NMR: $CDCl_3$: δ 7.26).

3.5.7 Identification of contact pheromone components: Syntheses of candidate pheromone components

The syntheses of candidate pheromone components and synthetic intermediates are reported in the SI.

3.5.8 Identification of contact pheromone components: T-rod bioassays (general procedures)

The T-rod apparatus[37] (Fig. 3.1c) consisted of a horizontal beam (25 × 0.4 cm) and a vertical beam (30 × 0.4 cm) held together by labelling tape (3 × 1.9 cm, Fisher Scientific, Ottawa, ON, CA). A piece of filter paper (2 cm^2) was attached to each distal end of the horizontal beam. For each bioassay, an aliquot of web extract (in methanol), or a blend of synthetic candidate pheromone components, was applied to the randomly assigned treatment filter paper, whereas methanol was applied to the control filter paper. The solvent was allowed to evaporate for 1 min before the onset of a 15-min bioassay. A randomly selected naïve male spider was placed at the base of the vertical beam and the time he spent courting on each filter paper was recorded. In response to the presence of female-produced or synthetic pheromone on a filter paper, the male engaged in courtship, pulling silk with his hindlegs from his spinnerets and adding it to the paper. Sensing contact pheromone, the male essentially behaves as if he were courting on the web of a

female. On a web, the male engages in web reduction prior to copulation, a behaviour that entails cutting sections of the female's web with his chelicerae and wrapping the dismantled web bundle with his own silk pulled from his spinnerets[41,56]. Each T-rod apparatus was used only once. Replicates of experiments as part of specific research objectives were run in parallel to eliminate day effects on responses of spiders. The sample size for each experiment was set to 20, unless otherwise stated.

3.5.9 Identification of contact pheromone components: T-rod bioassays (specific experiments) (fall 2017; spring & summer 2018)

Experiment 1 (fall 2017) tested a synthetic blend of volatile compounds **5**–**11** unique to mature *S. grossa* females (Fig. 3.1c and Appendix Table 3.1) *vs* a solvent control. Parallel experiment 2 tested one web equivalent of virgin female web extract, followed by testing each of the 20 HPLC fractions in six replicates for the occurrence of courtship (spring 2018).

Parallel experiments 3–6 (summer 2018) tested web extract at one female web equivalent (1 FWE) (Exp. 3), a ternary blend of the candidate contact pheromone components **12**, **16** and **17** (Fig. 3.2d, Exp. 4), the same ternary blend (**12**, **16**, **17**) in combination with the volatile compounds **5**–**11** (Exp. 5), and **5**–**11** on their own (Exp. 6).

Parallel dose-response experiments 7–11 (summer 2018) tested the ternary blend of **12**, **16**, and **17** at five FWEs: 0.001 (Exp. 7); 0.01 (Exp. 8); 0.1 (Exp. 9); 1.0 (Exp. 10); and 10 (Exp. 11).

Parallel experiments 12–15 tested the ternary blend, and all possible binary blends, of **12**, **16**, and **17**. Parallel experiments 16–18 tested **12** and **16** in binary combination (Exp. 16) and singly (Exps. 17, 18).

3.5.10 Origin of contact pheromone components (fall 2020)

To trace the origin of contact pheromone component **12** (and coeluting **16**), cold-euthanized female spiders were dissected in saline solution[55] (25 mL of water and 25 mL of methanol, 160 mM NaCl, 7.5 mM KCl, 1 mM $MgCl_2$, 4 mM $NaHCO_3$, 4 mM $CaCl_2$,

20 mM glucose, pH 7.4). Samples were homogenized (Kimble Pellet Pestle Motor, Kimble Kontes, USA) in methanol for 1 min, kept 24 h at room temperature for pheromone extraction, and then centrifuged (12,500 rpm, 3 °C for 20 min; Hermle Z 360 K refrigerated centrifuge; B. Hermle AG, Wehingen, DE) to obtain the supernatant for HPLC-MS analyses (see above) for the presence of **12** & **16**. Three sequential sets of dissections aimed to determine the (1) pheromone-containing body tagma, (2) the pheromone-containing tissues or glands in that tagma, and (3) the specific gland or tissue producing **12** & **16**.

To identify the pheromone-containing tagma, 11 spiders were severed at the pedicel, generating two tagmata: the cephalothorax with four pairs of legs and the abdomen. Each tagma was then extracted separately in 100 µL of methanol. Eight of 11 abdomen samples contained **12** & **16**, whereas only one of 11 thorax sample contained **12** & **16** (Exp. 19), albeit at only trace amounts.

With **12** & **16** being present in the abdomen, 20 additional abdomens were dissected[68] to obtain separate samples of (*i*) hemolymph (25 µL), (*ii*) ventral cuticle (~0.5 cm^2 near the pedicel, (*iii*) the ovaries, (*iv*) all silk glands combined, and (*v*) the gut (with anus, cloaca and Malpighian tubules). Remaining spider tissues (*vi*) were pooled as one sample, and 20 µL of the dissection buffer solution (*vii*) was obtained to detect potential pheromone bleeding. To each tissue sample, 50 µL of methanol were added. Only silk gland samples contained **12** & **16** (Exp. 20).

Having established that only silk gland samples contained **12** &**16**, the silk glands of 30 additional spiders were excised in the following order: (*i*) major ampullate gland, (*ii*) minor ampullate gland, (*iii*) anterior aggregate gland, (*iv*) posterior aggregate gland, (*v*) tubiliform, (*vi*) aciniform and flagelliform glands combined, and (*vii*) pyriform gland. The glands from three spiders were combined in each sample and extracted in 30 µL methanol. Seven of 10 posterior aggregate gland samples contained **12** & **16**, with other silk gland samples not containing **12** & **16** or in only trace amounts (Exp. 21).

3.5.11 Transition of contact pheromone components to mate attractant pheromone components: Evidence for hydrolysis of contact pheromone components (**12**, **16**, **17**) (spring 2021)

To test for the hydrolysis of the contact pheromone components **12, 16** and **17**, we compared their breakdown ratio (**18 /** (**12** + **16** + **17 + 18**) on independent webs aged 0 days and 14 days (Exp. 24). Each of 140 spiders was allowed to spin a web on bamboo scaffolds for three days. Then, the spiders were removed and webs – by random assignment – were extracted immediately (0-day-old webs) or after 14 days of aging (14-day-old webs). On each web, the amount of contact pheromone components **12**, **16** and **17**, and of amide **18** as a breakdown product, was quantified using HPLC-MS, with **12** and **18** at 25 ng/µL and 50 ng/µL as external standards.

3.5.12 Transition of contact pheromone components to mate attractant pheromone components: Y-tube olfactometer bioassays (general procedures)

Attraction of male spiders to web extracts and to candidate mate attractant pheromone components was tested in Y-tube olfactometers[56] (Fig. 3.4a) lined with bamboo sticks to provide grip for the bioassay spider. Test stimuli were presented in translucent oven bags (30 × 31 cm; Toppits, Mengen, DE) secured to the orifice of side-arms. Test stimuli consisted of a triangular bamboo prism scaffold (each side 8.5 cm long) bearing a spider's web, or bearing artificial webbing[30] (40 ± 2 mg; Bling Star, CN) that was treated with web extract or synthetic chemicals in methanol (100 µL) as the treatment stimulus or with methanol (100 µL) as the control stimulus. For each experimental replicate, a male spider was introduced into a glass holding tube and allowed 2 min to acclimatize. Then, the holding tube was attached via a glass joint to the Y-tube olfactometer, and an air pump was connected to the holding tube, drawing air at 100 mL/min through the olfactometer. Air entered the olfactometer through a glass tube secured to the oven bags' second opening. A male that entered the olfactometer within the 5-min bioassay period was classed a responder and his first choice of oven bag (the oven bag he reached first) was recorded. Whenever a set of 30 replicates was completed by the same observer, using 30 separate Y-tubes, the Y-tubes were cleaned with hot water

and soap (Sparkleen, Thermo Fisher Scientific, MA, United States) and dried in an oven at 100 °C for three hours, whereas the bamboo sticks and the oven bags were discarded.

3.5.13 Transition of contact pheromone components to mate attractant pheromone components: Y-tube olfactometer bioassays (specific experiments) (summer 2018)

In experiment 22, 23, 25–27, males were offered a choice between a solvent control stimulus and a treatment stimulus. The treatment stimulus consisted of (*i*) virgin female web-extract (1 web-equivalent) (Exp. 22, N = 24), (*ii*) the volatile compounds **5**–**11** unique to sexually mature females (Fig. 3.1d) (Exp. 23, N = 24), (*iii*) all breakdown products of the contact pheromone components **12, 16** and **17**, consisting of the amide *N*-4-methylvaleroyl-*L*-serine (**18**) and the corresponding carboxylic acids **19, 20** and **21** (Exp. 25, N = 30)**,** (*iv*) a blend of the acids **19, 20** and **21** (Exp. 26, N = 30), and (*v*) the amide **18** as a single compound (Exp. 27, N = 30). Compounds were tested at quantities as determined in virgin female web extract (50 webs in 150 µL of dichloromethane), following silyl-ester derivatization[69] of acids in the extract, with valeric acid (200 ng; ≥99%, Sigma Aldrich, St. Louis, USA) added as an internal standard. Per web equivalent, there were 103 ng of **19**, 3 ng of **20** and 54 ng of **21**. The amide **18** was present at 200 ng per web equivalent, as determined using *N*-3-methylbutnaoyl-*L*-serine methyl ester as an external standard.

3.5.14 Transition of contact pheromone components to mate attractant pheromone components: Hallway of buildings experiment (fall 2018)

As the ternary blend of the carboxylic acids **19, 20** and **21** attracted male spiders in Y-tube olfactometers (see Results), we aimed to confirm their functional role as mate attractant pheromone components also in ‘field’ settings (Exp. 28). To this end, we set up 10 replicates of paired traps in building hallways on the Burnaby campus of Simon Fraser University. Adhesive-coated traps (Bell Laboratories Inc., Madison, WI, USA) were spaced 0.5 m within pairs and 20 m between pairs. By random assignment, one trap in each pair was baited with the carboxylic acids **19, 20** and **21** formulated in 200 µL of mineral oil (Anachemia, Montreal, CA; 2.8 mg of **19**, 0.112 mg of **20,** and 1.52 mg of **21**), whereas the control trap received mineral oil only. Test stimuli were disseminated

from a 400-µL microcentrifuge tube (Evergreen Scientific, Ranco Dominguez, CA, USA) with a hole in its lid punctured by a No. 3 insect pin (Hamilton Bell, Montvale, NJ, USA). Every week for four months (September to December 2018), traps were checked, lures replaced, and the position of the treatment and the control trap within each trap pair was re-randomized.

3.5.15 Communication function of amide breakdown product 18 (fall 2018)

As the amide **18** did not attract males in Y-tube olfactometer experiments (see Results), we tested its alternate potential function as a contact pheromone component which, if active, would induce courtship by males. Using the T-rod apparatus (Fig. 3.1c), we treated one piece of filter paper with a solvent control and the other with a blend comprising both the contact pheromone components **12, 16** and **17** and the amide **18** (Exp. 29), a blend of **12, 16** and **17** (Exp. 30), and **18** alone (Exp. 31).

3.5.16 Mechanisms underlying the transition of contact pheromone components to mate attractant pheromone components: Relationship between web pH and breakdown rates of contact pheromone components (summer 2020)

We allowed each of 70 spiders to spin two webs, using one web to quantify the amide breakdown product (**18**) of the contact pheromone components (see above), and the other web to determine its pH according to the slurry method[57] (Exp. 32). To this end, we first measured the pH of 50 µL water (HPLC grade, EMD Millipore Corp., Burlington, MA, USA) and then a web with the water functioning as a conductor for the pH meter (LAQUAtwin pH 22 (Horiba, Kyoto, JP). Between web measurements, the pH meter was rinsed with water and regularly re-calibrated using a pH 7 and a pH 4 buffer (Horiba, Kyoto, JP).

3.5.17 Mechanisms underlying the transition of contact pheromone components to mate attractant pheromone components: Testing for pH-dependent saponification of contact pheromone components (**12**, **16**, **17**) (summer 2021)

To test whether pH alone catalyses saponification of the ester-bond of contact pheromone components (**12**, **16**, **17**), synthetic **12** was added to a 40% aqueous pH 7

buffer solution (Exp. 34), a pH 4 buffer solution (Exp. 34), and to acetonitrile (Exp. 35) as a polar aprotic solvent control (N =12; 100 ng/µL each). pH-Dependent breakdown of **12** over time was assessed by analysing (HPLC-MS) diluted aqueous aliquots (2.5 ng/µl) of each sample at day 0 and after 14 days of storage at room temperature.

3.5.18 Mechanisms underlying the transition of contact pheromone components to mate attractant pheromone components: Testing for the presence of a carboxylesterhydrolase (CEH) (summer 2021)

To test for the presence of a carboxylesterhydrolase (CEH), for each of three replicates we extracted (*i*) five webs of adult virgin female *L. hesperus* (positive control, known to have a CEH[45]), (*ii*) 20 webs of subadult *S. grossa* (deemed to have not yet produced a CEH), and (*iii*) 10 webs of adult virgin female *S. grossa,* accounting for the different amounts of silk produced by these three groups of spiders. For each replicate, webs were extracted in 200 µL 0.05 M Sørensen buffer[58] and analysed by Bioinformatics Solutions (Waterloo, ON, CA). After web samples were incubated 20 min at 60 °C in 2× sample volumes of 10% SDS (lauryl sulfate; protein-denaturing anionic detergent), they were sonicated 20 min. Then, the supernatant was withdrawn, reduced with dithiothreitol (DTT), and alkylated with iodoacetamide (IAA). Alkylated samples were treated further with a *protein* solvent (S-Trap kit; Protifi, Farmingdale, NY, USA). Briefly, samples were acidified by phosphoric acid to pH ≤ 1. Then 6× of sample volume S-trap buffer was mixed in. The mixture was loaded by centrifugation onto a S-Trap Micro Spin Column and washed 3× with S-trap buffer. Using the serine protease trypsin, protein digestions were carried out at 47 °C for 1 h in 50 mM triethylamonium bicarbonate (TEAB) buffer within the S-Trap Micro Spin column. Digestion products were eluted sequentially with 40 µL 50 mM TEAB and 0.2% formic acid. Eluates were dried and re-suspended in 0.1% formic acid.

Eluates were analysed by HPLC-MS/MS in positive ion mode on a Thermo Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer (ThermoFisher, San Jose, CA, USA), equipped with a nanospray ionization source and a Thermo Fisher Ultimate 3000 RSLCnano HPLC System (ThermoFisher). Peptide mixtures were loaded onto a

PEPMAP100 C_{18} trap column (75 µm × 20 mm, 5 µm particle size; ThermoFisher) at a constant flow of 30 µL/min and 60 °C isothermal. Peptides were eluted at a rate of 0.2 µL/min and separated using a Reprosil C_{18} analytical column (75 µm × 15 mm, 1.9 µm particle size; PepSep, DK) with a 60-min solvent gradient: 0–45 min: 4–35% acetonitrile + 0.1% formic acid; 45–55 min: 90% acetonitrile + 0.1% formic acid; 55–60 min: 4% acetonitrile + 0.1% formic acid.

MS data were acquired in data-dependent mode with a cycle time of 3 s. MS1 scan data were acquired with the Orbitrap mass analyzer, using a mass range of 400–1,600 *m/z,* with the resolution set to 120,000. The automatic gain control (AGC) was set to 4e5, with a maximum ion injection time of 50 ms, and the radio frequency (RF) lens set to 30%. Isolations for MS2 scans were run using a quadrupole mass analyzer, with an isolation window of 0.7. MS2 scan data were acquired with the Orbitrap mass analyzer at a resolution of 15,000 *m/z*, with a maximum ion injection time of 22 ms, and the AGC target set to 5e4. Higher energy collisional dissociation (HCD; fixed normalized collision energy: 30%) was used for generating MS2 spectra, with the number of microscans set to 1.

3.5.19 Statistics and Reproducibility

Data (Appendix Table 3.2) were analysed statistically using R[70]. Data of experiments 1–18 and 29–31 (testing courtship by male spiders in response to contact pheromone components) were analysed with a Wilcoxon test or Kruskal-Wallis two-tailed rank sum test with Benjamini-Hochberg correction to adjust for multiple comparison. Data of experiments 19–21 (revealing the presence of contact pheromone components in the abdomen, silk glands, and posterior aggregate silk gland) were analysed with two-tailed, rather than one-tailed, Wilcoxon test or Kruskal-Wallis rank tests because we had no strong assumption as to whether or not pheromone would be present in any of these potential pheromone sources. The p-values were adjusted for multiple comparison using the Benjamini-Hochberg method. Y-tube olfactometer data of experiments 22, 23, 25–27 as well as the hallway experiment 28 (revealing attraction of male spiders to volatile pheromone components) were analysed using an one-tailed[71]

binomial test, anticipating attraction of spiders to volatile mate attractant pheromone components rather than to solvent control stimuli. Data of experiment 32 (revealing a correlation between web pH and breakdown of web-borne contact pheromone components) were analysed using generalized linear models. Data of experiments 33–35 (showing pH-dependent breakdown of synthetic contact pheromone) were compared using a two-tailed Kruskal-Wallis test with Benjamini-Hochberg correction.

3.6 Acknowledgments

We thank three anonymous reviewers for constructive comments, Hongwen Chen, Adam Blake and Catherine McCaughey for technical advice, and Stephen Takács and Asim Renyard for preparing figures 3.3 and 3.4a respectively. Further we thank our funders: AF: Graduate Fellowship, McCarthy Bursary from Simon Fraser University, Alexander Graham Bell Scholarship from the Natural Sciences and Engineering Research Council of Canada (NSERC); GG: NSERC – Industrial Research Chair with Scotts Canada Ltd. and BASF Canada as the industrial sponsors. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

3.7 References

1. Darwin, C. *The Descent of Man and Selection in Relation to Sex*. (Random House, Modern Library, 1871).
2. Clutton-Brock, T. Sexual selection in males and females. *Science.* **318**, 1882–1885 (2007).
3. Emlen, D. J., Warren, I. A., Johns, A., Dworkin, I. & Lavine, L. C. A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. *Science.* **337**, 860–864 (2012).
4. Allen, B. J. & Levinton, J. S. Costs of bearing a sexually selected ornamental weapon in a fiddler crab. *Funct. Ecol.* **21**, 154–161 (2007).
5. Dabelsteen, T. *et al.* Quiet song in song birds: An overlooked phenomenon. *Bioacoustics* **9**, 89–105 (1998).
6. Cocroft, R. B., Gogala, M., Hill, P. S. M. & Wessel, A. *Studying vibrational communication*. vol. 3 (2014).
7. Ando, T., Inomata, S. & Yamamoto, M. Lepidopteran sex pheromones. *Top. Curr. Chem.* **239**, 51–96 (2004).
8. Breithaupt, T. & Thiel, M. *Chemical communication in Crustaceans*. (Springer

Science+Business Media, 2011).
9. Varner, E., Gries, R., Takács, S., Fan, S. & Gries, G. Identification and field testing of volatile components in the sex attractant pheromone blend of female house mice. *J. Chem. Ecol.* **45**, 18–27 (2019).
10. Littlewood, P. M. H. The chemosensory behaviour of *Lithobius forficatus* (Myriapoda: Chilopoda). 2. Bioassay and chemistry of the coxal pheromone. *J. Zool.* **215**, 523–535 (1988).
11. Beauche, F. & Richard, F.-J. The best timing of mate search in *Armadillidium vulgare* (Isopoda, Oniscidea). *PLoS One* **7**, e57737 (2013).
12. Ayasse, M., Paxton, R. J. & Tengö, J. Mating behavior and chemical communication in the order hymenoptera. *Annu. Rev. Ecol. Evol. Syst.* **46**, 31–78 (2001).
13. Symonds, M. R. E. & Elgar, M. A. The evolution of pheromone diversity. *Trends Ecol. Evol.* **23**, 220–228 (2008).
14. Francke, W. & Schulz, S. Pheromones of Terrestrial Invertebrates. in *Comprehensive Natural Products II* (eds. Mander, L. & Liu, H.-W.) 153–223 (2010).
15. Ayasse, M., Stökl, J. & Francke, W. Chemical ecology and pollinator-driven speciation in sexually deceptive orchids. *Phytochemistry* **72**, 1667–1677 (2011).
16. Savoie, A., Borden, J. H., Pierce, H. D., Gries, R. & Gries, G. Aggregation pheromone of *Pityogenes knechteli* and semiochemical based interactions with three other bark beetles. *J. Chem. Ecol.* **24**, 321–337 (1998).
17. Billen, J. & Morgan, E. D. Sources and Secretions. in *Pheromone Communication in Social Insects* (eds. Meer, R. K. Vander, Breed, M. D., Espelie, K. E. & Winston, M. L.) 3–33 (Westview Pres, 1998). doi:10.2307/1313541.
18. Landoldt, P. J. & Heath, R. R. Alarm pheromone behavior of *Vespula squamosa*. *Florida Entomol.* **70**, 222–225 (1987).
19. Stowe, M. K., Turlings, T. C. J., Loughrin, J. H., Lewis, W. J. & Tumlinson, J. H. The chemistry of eavesdropping, alarm, and deceit. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 23–28 (1995).
20. Witzgall, P., Kirsch, P. & Cork, A. Sex pheromones and their impact on pest management. *J. Chem. Ecol.* **36**, 80–100 (2010).
21. Steiner, S., Hermann, N. & Ruther, J. Characterization of a female-produced courtship pheromone in the parasitoid *Nasonia vitripennis*. *J. Chem. Ecol.* **32**, 1687–1702 (2006).
22. Smadja, C. & Butlin, R. K. On the scent of speciation: The chemosensory system and its role in premating isolation. *Heredity (Edinb).* **102**, 77–97 (2009).
23. Ma, T. *et al.* Analysis of tea geometrid (*Ectropis grisescens*) pheromone gland extracts using GC-EAD and GC×GC/TOFMS. *J. Agric. Food Chem.* **64**, 3161–3166 (2016).
24. Chemnitz, J., Bagrii, N., Ayasse, M. & Steiger, S. Variation in sex pheromone emission does not reflect immunocompetence but affects attractiveness of male burying beetles—a combination of laboratory and field experiments. *Sci. Nat.* **104**, (2017).
25. Vosshall, L. B., Amrein, H., Morozov, P. S., Rzhetsky, A. & Axel, R. A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell* **96**, 725–736

(1999).
26. Yuvaraj, J. K. *et al.* Putative ligand binding sites of two functionally characterized bark beetle odorant receptors. *BMC Biol.* **19**, 1–21 (2021).
27. Hou, X.-Q. *et al.* Functional evolution of a bark beetle odorant receptor clade detecting monoterpenoids of different ecological origins. *Mol. Biol. Evol.* **38**, 4934–4947 (2021).
28. Arn, H., Städler, E. & Rauscher, S. The electroantennographic detector—a selective and sensitive tool in the gas chromatographic analysis of insect pheromones. *Z. Naturforsch* **30c**, 722–725 (1975).
29. Gries, R. *et al.* Limoniic acid - Major component of the sex pheromones of the click beetles *Limonius canus* and *L. californicus*. *J. Chem. Ecol.* **47**, 123–133 (2021).
30. Fischer, A., Hung, E. & Gries, G. Female false black widow spiders, *Steatoda grossa*, recognize webs based on physical and chemical cues. *Entomol. Exp. Appl.* **167**, 803–810 (2019).
31. Schulz, S. Spider pheromones - a structural perspective. *J. Chem. Ecol.* **39**, 1–14 (2013).
32. Foelix, R. *Biologie der Spinnen*. (Chimaira, 2015).
33. Uhl, G. & Elias, D. Communication. in *Spider Behaviour* (ed. Herberstein, M. E.) 127–189 (Cambridge University Press, 2011).
34. Trabalon, M. & Assi-Bessekon, D. Effects of web chemical signatures on intraspecific recognition in a subsocial spider, *Coelotes terrestris* (Araneae). *Anim. Behav.* **76**, 1571–1578 (2008).
35. Fischer, A., Schulz, S., Ayasse, M. & Uhl, G. Pheromone communication among sexes of the garden cross spider *Araneus diadematus*. *Sci. Nat.* **108**, 1–11 (2021).
36. Scott, C. E., McCann, S. & Andrade, M. C. B. B. Male black widows parasitize mate-searching effort of rivals to find females faster. *Proc. R. Soc. B Biol. Sci.* **286**, 20191470 (2019).
37. Fischer, A., Lee, Y., Stewart, J. & Gries, G. Dodging sexual conflict? – Sub-adult females of a web-building spider stay cryptic to mate-seeking adult males. *Ethology* **124**, 838–843 (2018).
38. Trabalon, M., Bagneres, A. & Roland, C. Contact sex signals in two sympatric spider species, *Tegenaria domestica* and *Tegenaria pagana*. *J. Chem. Ecol.* **23**, 747–758 (1997).
39. Fischer, A. Chemical communication in spiders - a methodological review. *J. Arachnol.* **47**, 1–27 (2019).
40. Kasumovic, M. M. & Andrade, M. C. B. Discrimination of airborne pheromones by mate-searching male western black widow spiders (*Latrodectus hesperus*): species-and population-specific responses. *Can. J. Zool.* **82**, 1027–1034 (2004).
41. Scott, C., Gerak, C., McCann, S. & Gries, G. The role of silk in courtship and chemical communication of the false widow spider, *Steatoda grossa* (Araneae: Theridiidae). *J. Ethol.* **36**, 191–197 (2018).
42. Schulz, S. & Toft, S. Identification of a sex pheromone from a spider. *Science.* **260**, 1635–1637 (1993).
43. Jerhot, E., Stoltz, J. A., Andrade, M. C. B. & Schulz, S. Acylated serine derivatives: A unique class of arthropod pheromones of the Australian Redback

spider, *Latrodectus hasselti*. *Angew. Chemie* **49**, 2037–2040 (2010).
44. Scott, C., McCann, S., Gries, R., Khaskin, G. & Gries, G. *N*-3-methylbutanoyl-*O*-methylpropanoyl-L-serine methyl ester – pheromone component of western black widow females. *J. Chem. Ecol.* **41**, 465–472 (2015).
45. Chaw, R. C., Correa-Garhwal, S. M., Clarke, T. H., Ayoub, N. A. & Hayashi, C. Y. Proteomic evidence for components of spider silk synthesis from black widow silk glands and fibers. *J. Proteome Res.* **14**, 4223–4231 (2015).
46. Bugg, T. *Introduction to Enzyme and Coenzyme Chemistry*. *Introduction to Enzyme and Coenzyme Chemistr* (Blackwell Publishing, 2012). doi:10.1002/9781444305364.
47. Heim, M., Keerl, D. & Scheibel, T. Spider silk: From soluble protein to extraordinary fiber. *Angew. Chemie - Int. Ed.* **48**, 2–15 (2009).
48. Bradley, R. A. *Common Spiders of North America*. (University of California Press, 2012).
49. Bellmann, H. *Der Kosmos-Spinnenführer*. (Kosmos, 2010).
50. Liu, J., May-Collado, L. J., Pekár, S. & Agnarsson, I. A revised and dated phylogeny of cobweb spiders (Araneae, Araneoidea, Theridiidae): A predatory cretaceous lineage diversifying in the era of the ants (Hymenoptera, Formicidae). *Mol. Phylogenet. Evol.* **94**, 658–675 (2016).
51. Wheeler, W. C. *et al.* The spider tree of life: Phylogeny of araneae based on target-gene analyses from an extensive taxon sampling. *Cladistics* **33**, 574–616 (2017).
52. Garb, J. E. & Hayashi, C. Y. Molecular evolution of alpha-latrotoxin, the exceptionally potent vertebrate neurotoxin in black widow spider venom. *Mol. Biol. Evol.* **30**, 999–1014 (2013).
53. Hashimoto, N., Aoyama, T. & Shioiri, T. New methods and reagents in organic synthesis. 14. A simple efficient preparation of methyl esters with trimethylsilyldiazomethane (TMSCHN2) and its application to gas chromatographic analysis of fatty acids. *Chem. Pharm. Bull.* **29**, 1475–1478 (1981).
54. Schulz, S. Semiochemistry of spiders. in *Advances in Insect Chemical Ecology* (eds. Carde, R. T. & Millar, J. G.) 110–150 (Cambridge University Press, 2004). doi:10.1017/cbo9780511542664.005.
55. Groome, J. R., Townley, M. A., de Tschaschell, M. & Tillinghast, E. K. Detection and isolation of proctolin-like immunoreactivity in arachnids: Possible cardioregulatory role for proctolin in the orb-weaving spiders *Argiope* and *Araneus*. *J. Insect Physiol.* **37**, 9–19 (1991).
56. Fischer, A. *et al.* Multimodal and multifunctional signaling? – Web reduction courtship behavior in a North American population of the false black widow spider. *PLoS One* **15**, e0228988 (2020).
57. Pudipeddi, M. *et al.* Measurement of surface pH of pharmaceutical solids: A critical evaluation of indicator dye-sorption method and its comparison with slurry pH method. *J. Pharm. Sci.* **97**, 1831–1842 (2008).
58. Babczyńska, A., Wilczek, G. & Migula, P. Effects of dimethoate on spiders from metal pollution gradient. *Sci. Total Environ.* **370**, 352–359 (2006).
59. Robertson, D. H. L., Beynon, R. J. & Evershed, R. P. Extraction, characterization, and binding analysis of two pheromonally active ligands associated with major

urinary protein of house mouse (*Mus musculus*). *J. Chem. Ecol.* **19**, 1405–1416 (1993).
60. Armstrong, S. D., Robertson, D. H. L., Cheetham, S. A., Hurst, J. L. & Beynon, R. J. Structural and functional differences in isoforms of mouse major urinary proteins: A male-specific protein that preferentially binds a male pheromone. *Biochem. J.* **391**, 343–350 (2005).
61. Dicko, C., Vollrath, F. & Kenney, J. M. Spider silk protein refolding is controlled by changing pH. *Biomacromolecules* **5**, 704–710 (2004).
62. Vollrath, F., Knight, D. P. & Hu, X. W. Silk production in a spider involves acid bath treatment. *Proc. R. Soc. B Biol. Sci.* **265**, 817–820 (1998).
63. Holdcraft, R., Rodriguez-Saona, C. & Stelinski, L. L. Pheromone autodetection: Evidence and implications. *Insects* **7**, 1–29 (2016).
64. Umbers, K. D. L., Symonds, M. R. E. & Kokko, H. The mothematics of female pheromone signaling: Strategies for aging virgins. *Am. Nat.* **185**, 417–432 (2015).
65. Jackson, B. D. & Morgan, E. D. Insect chemical communication: Pheromones and exocrine glands of ants. *Chemoecology* **4**, 125–144 (1993).
66. Gu, S. H. *et al.* Identification of genes expressed in the sex pheromone gland of the black cutworm *Agrotis ipsilon* with putative roles in sex pheromone biosynthesis and transport. *BMC Genomics* **14**, 636–357 (2013).
67. Van Den Dool, H. & Kratz, P. D. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J. Chromatogr. A* **11**, 463–471 (1963).
68. Chaw, R. C. & Hayashi, C. Y. Dissection of silk glands in the Western black widow. *J. Arachnol.* **46**, 159–161 (2018).
69. Stalling, D., L., C. W. G. & Zumwalt, R. W. A new silylation reagent for amino acids bis (trimethylsilyl) trifluoroacetamide (BSTFA). *Biochem. Biophys. Res. Commun.* **31**, 616–622 (1968).
70. R Core Team. *R: A language and environment for statistical computing*. (R Foundation for Statistical Computing, 2020).
71. Ruxton, G. D. & Neuhäuser, M. When should we use one-tailed hypothesis testing? *Methods Ecol. Evol.* **1**, 114–117 (2010).

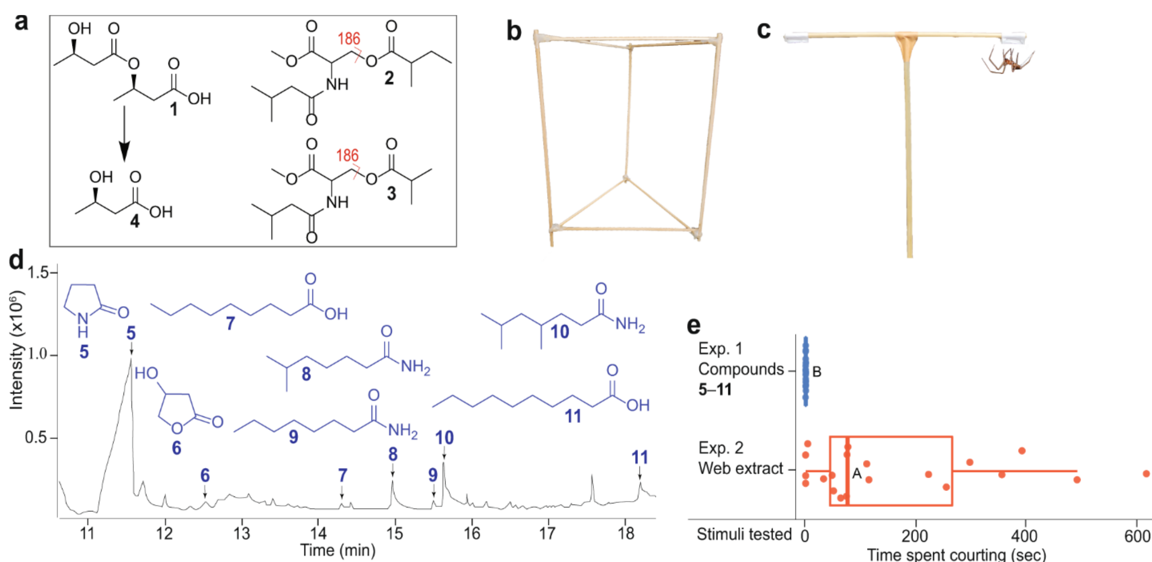


Figure 3.1: Known contact pheromone components of spiders and methods to identify analogous components produced by *Steatoda grossa* females. (**a**) Pheromone components of the spiders (*i*) *Linyphia triangularis* ([(*R*)-3-hydroxybutyryloxy-butyric acid (**1**) with its breakdown product (*R*)-3-hydroxybutyric acid (**4**)], (*ii*) *Latrodectus hasselti* ([*N*-3-methyl-butyryl-*O*-(*S*)-2-methylbutyryl-*L*-serine methyl ester (**2**)], and (*iii*) *Latrodectus hesperus* [(*N*-3-methylbutanoyl-*O*-methylpropanoyl-*L*-serine methyl ester (**3**)]. (**b**) Triangular prism scaffold for a female spider to build her web. (**c**) T-rod apparatus for testing courtship behaviour by *S. grossa* males in response to test stimuli (web extract or fractions thereof; synthetic candidate phseromone components; solvent control) applied to a piece of filter paper attached to each distal end of the horizontal arm. (**d**) Total ion chromatogram of compounds unique to sexually mature *S. grossa* females (pyrrolidin-2-one (**5**), 4-hydroxyhydrofuran-2(3*H*)-one (**6**), nonanoic acid (**7**), dodecanoic acid (**8**), 6-methylheptanamide (**9**), octanamide (**10**), 4,6-dimethyl heptanamide (**11**)) identified by gas chromatography-mass spectrometry of crude female web extract. (**e**) Extent of courtship by *S. grossa* males in response to female web extract or synthetic candidate pheromone components. Circles and boxplots show the time single male spiders courted in each replicate and the distribution of data (minimum, first quartile, median, third quartile, maximum), respectively. Medians with different letters indicate statistically significant differences in courtship responses. Wilcoxon test, $P < 0.05$.

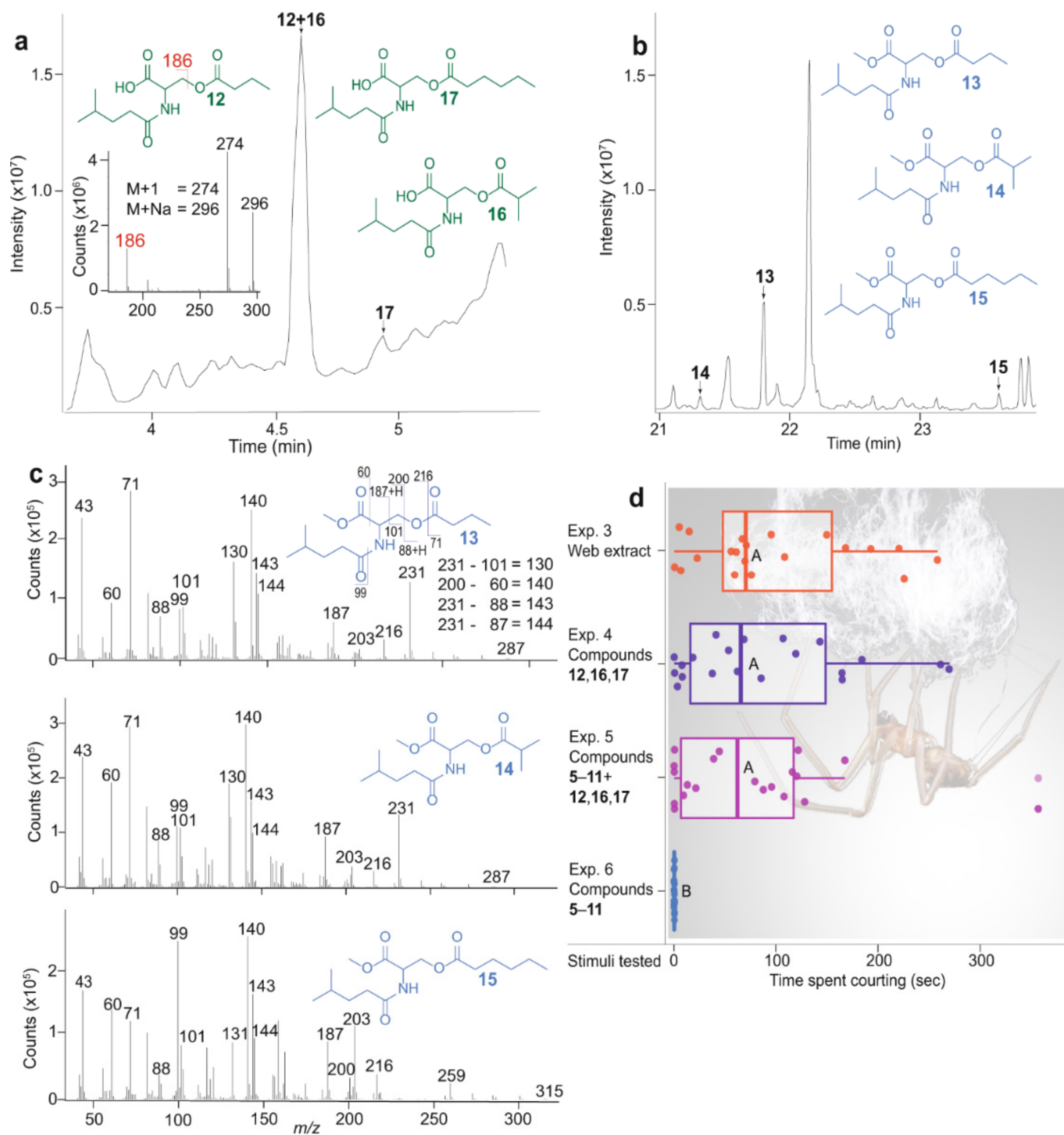


Figure 3.2: **Contact pheromone components of female *Steatoda grossa*.** (**a**) High performance liquid chromatogram (HPLC) of compounds [*N*-4-methylvaleroyl-*O*-isobutyroyl-*L*-serine (**12**); *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**16**); *N*-4-methylvaleroyl-*O*-hexanoyl-*L*-serine (**17**)] present in crude web extract of female *S. grossa,* and HPLC mass spectrum of **12** (with **16** coeluting). (**b, c**) Total ion chromatogram (**b**) and mass spectra (**c**) of compounds [*N*-4-methylvaleroyl-*O*-butyroyl-*L*-serine methyl ester (**13**), *N*-4-methylvaleroyl-*O*-isobutyroyl-*L*-serine methyl ester (**14**), *N*-4-methylvaleroyl-*O*-hexanoyl-*L*-serine methyl ester (**15**)] identified by gas chromatography-mass spectrometry (GC-MS) in esterified web extract of female *S. grossa*. (**d**) Extent of courtship by male *S. grossa* in response to stimuli tested in T-rod bioassays. The names of

compounds 5–11 are reported in the caption of Fig. 3.1. Circles and boxplots show the time single male spiders courted in each replicate and the distribution of data (minimum, first quartile, median, third quartile, maximum), respectively. Medians with different letters indicate statistically significant differences in courtship responses; Kruskal-Wallis χ^2 test with Benjamini-Hochberg correction to account for multiple comparisons, $P < 0.05$.

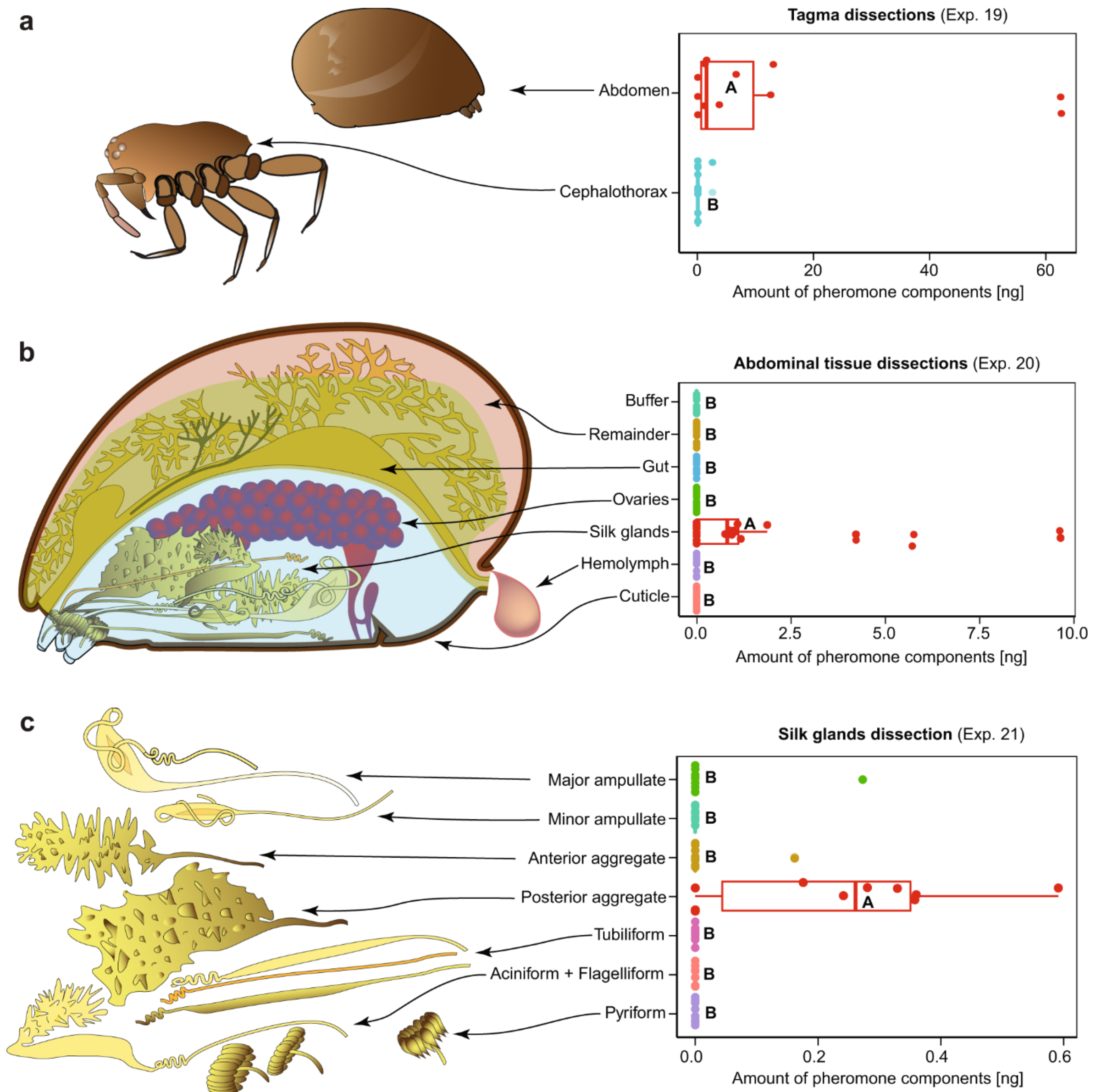


Figure 3.3: **Origin of contact pheromone components produced by female *Steatoda grossa*.** (**a**) High performance liquid chromatography - mass spectrometry (HPLC-MS) quantification of two contact pheromone components [*N*-4-methylvaleroyl-*O*-isobutyroyl-*L*-serine (**12**) coeluting with *N*-4-methylvaleroyl-*O*-isobutyroyl-*L*-serine (**16**)], present in the abdomen and cephalothorax of female spiders. (**b**) HPLC-MS quantification of **12** & **16** in the hemolymph and various tissues of the abdomen. (**c**) HPLC-MS quantification of **12** & **16** in various silk glands. In each of experiments 19–21, circles and boxplots show the amount of **12** & **16** present in each spider and the distribution of data (minimum, first quartile, median, third quartile, maximum), respectively. Medians with different letters indicate significantly different amounts of **12** & **16** present

in various sources; Wilcoxon and Kruskal-Wallis χ^2 test with Benjamini-Hochberg correction to account for multiple comparisons, $P < 0.05$.

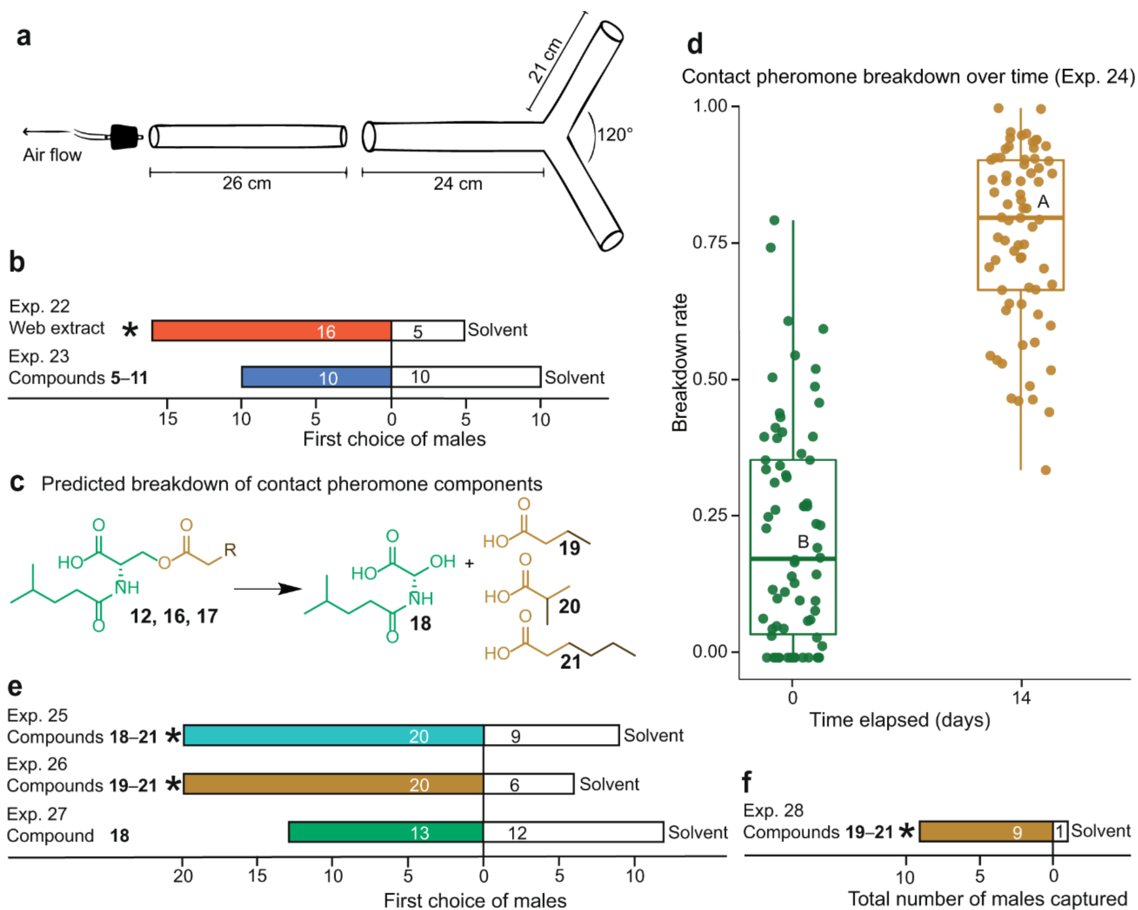


Figure 3.4: Transition of contact pheromone components produced by female *Steatoda grossa* to sex attractant pheromone components. (**a**) Moving-air dual-choice Y-tube olfactometer. (**b**) Attraction of *S. grossa* males in Y-tube olfactometers to extracts of female webs and to volatile compound **5**–**11** (names in Fig. 3.1 caption) unique to sexually mature females. (**c**) Predicted breakdown of contact pheromone components **12**, **16**, and **17** to the amide **18** and the volatile carboxylic acid mate attractant pheromone components **19**, **20**, and **21**. (**d**) Breakdown rate of contact pheromone components [ratio of **18** / (**12** + **16** + **17** + **18**)] on webs extracted 0 or 14 days after being built; circles and boxplots show the breakdown rates of single webs and the distribution of data (minimum, first quartile, median, third quartile, maximum), respectively, at days 0 and 14, which differed significantly (Wilcoxon test, P <0.05). (**e**) Attraction of *S. grossa* males in Y-tube olfactometers to single- or multiple-component blends of synthetic compounds; in each experiment, an asterisk denotes a significant preference for the treatment stimulus (one-tailed binomial tests; P <0.05). (**f**) Captures of *S. grossa* males in 10 pairs of sticky traps that were deployed in building hallways between September and December 2018. During weekly checks, the position of the treatment and control trap within each pair was randomized; the treatment trap was baited with the carboxylic acids **19**, **20**, and **21** (see Methods for detail), whereas the

control trap was left unbaited; the asterisk denotes a significant preference for the treatment trap (one-tailed binomial test; $P < 0.05$).

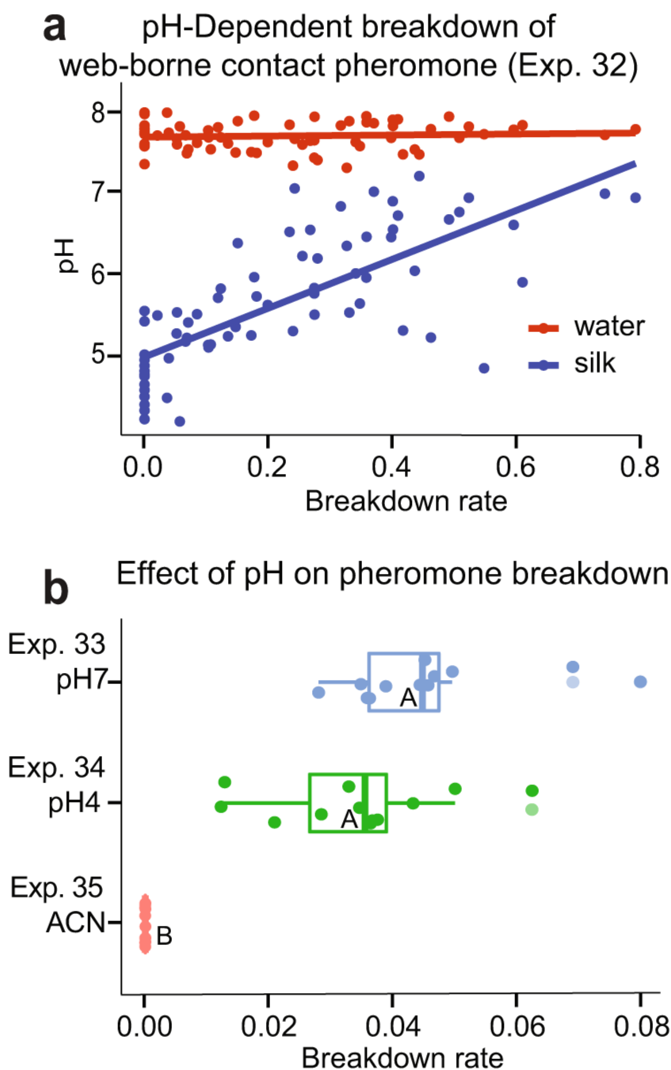


Figure 3.5: **pH-dependent breakdown of contact pheromone components**. (**a**) Relationship between the pH of female *Steatoda grossa* webs and the breakdown rate of contact pheromone components **12**, **16** and **17**, calculated as ratio of **18** / (**12** + **16** + **17** + **18**) (in blue); control measurements of the water's pH are displayed in red. (**b**) Effect of pH on breakdown of synthetic contact pheromone component **12**, calculated as the ratio of **18** / (**12**+**18**). Circles and boxplots show the breakdown rate of each sample and the distribution of data (minimum, first quartile, median, third quartile, maximum), respectively, at pH 4 and pH 7; medians with the same letter indicate no significant difference in breakdown rates (Kruskal-Wallis test; $P < 0.05$). Note the different scales of the x-axis in subpanels **a** and **b**; **12** = *N*-4-methylvaleroyl-*O*-isobutyroyl-*L*-serine; **18** = *N*-4-methylvaleroyl-*L*-serine (amide), ACN = acetonitrile.

Chapter 4: Non-targeted metabolomics aids in sex pheromone identification – a proof-of-concept study with the triangulate cobweb spider, *Steatoda triangulosa*.[1]

[1]A very similar version of this chapter will be submitted for peer-review, with a target journal not yet decided. The corresponding manuscript will have the following authors: Andreas Fischer, Andrea C. Roman-Torres, Jane Vurdela, Yerin Lee, Nastaran Bahar, Regine Gries, Santosh Alamsetti, Hongwen Chen, and Gerhard Gries.

4.1 Abstract

Targeted metabolomics has been widely used in pheromone research but may miss pheromone components in study organisms that produce pheromones in trace amount and/or lack bio-detectors (e.g., antennae) to readily locate them in complex samples. Here, we used non-targeted metabolomics – together with high-performance liquid chromatography-mass spectrometry (HPLC-MS), gas chromatography-MS, and behavioural bioassays – to unravel the sex pheromone of the triangulate cobweb spider, *Steatoda triangulosa*. A ternary blend of three contact pheromone components [*N*-4-methylvaleroyl-*O*-isobutyryl-L-serine (**5**), *N*-3-methyl-butyryl-*O*-propionyl-L-serine (**11**), and *N*-3-methyl-butyryl-*O*-butyroyl-L-serine (**12**)] elicited courtship by *S. triangulosa* males as effectively as female web extract. Hydrolysis of **5**, **11** and **12** at the ester bond gave rise to two mate-attractant pheromone components [butyric acid (**7**) and isobutyric acid (**8**)] which attracted *S. triangulosa* males as effectively as female webs. Pheromone components **11** and **12** were reported in spiders for the first time, and were discovered only using non-targeted metabolomics and GC-MS. All compounds resemble pheromone components previously identified in widow spiders. Our study provides impetus to apply non-targeted metabolomics for pheromone research in a wide range of animal taxa.

4.2 Introduction

Sexually reproducing organisms commonly attract or locate mates through sexual communication signals. Signals may be uni-, bi- or poly-modal with visual, chemical, acoustic, vibratory, and tactile characteristics.[1,2] Pheromones are thought to be the oldest type of sexual communication signals.[3] They are chemicals, or blends of chemicals, released by a signaller that causes a response by conspecific signal recipients.[4,5] Pheromones are prevalent in many animal taxa,[6] including insects,[7–10] myriapods,[11] crustaceans,[12] fish,[13] and mammals,[14] and may be airborne or substrate-borne and be sensed by olfactory receptors[15] or contact chemoreceptors.[16] Regardless of their physicochemical characteristics, pheromone components commonly occur in complex blends of analyte and are not easily located, isolated, and identified.[17]

Metabolomics entails the systematic identification and quantitation of metabolites, and their changes over time, in biological samples. Analytical techniques include, but are not limited to, gas chromatography - mass spectrometry (GC-MS), and high-performance liquid chromatography - mass spectrometry (HPLC-MS). The choice of analytical technique is based, in part, on chemical characteristics of target compounds and their abundance in samples. During GC-MS and HPLC-MS analyses, chemicals are separated and broken into mass fragments (ions), resulting in a mass spectrum for each sufficiently abundant compound and in a total ion chromatogram (TIC) that is created by summing up intensities of all mass spectral peaks belonging to the same scan.[18] Mass spectra then provide information about the molecular structure of compounds.[18,19]

Comparative metabolomics in pheromone identification research compares analytes obtained from animals that were capable (e.g. sexually mature), or not (e.g. sexually immature), of producing pheromone.[17] Traditional *targeted* metabolomics compares peaks between TICs and focuses on peaks for pheromone identification that are visually unique in one type of analyte (Fig. 4.1a).[20–23] However, exclusive focus on visually unique peaks may miss pheromone components that co-elute with non-pheromonal compounds (Fig. 4.1b) or occur at trace quantities (Fig. 4.1c).

Pheromone research has focused on insects, with more than 3000 pheromones identified to date[8]. This remarkable progress is attributed to a landmark invention 53 years ago that combined gas chromatography with electrophysiology for insect pheromone analyses.[24,25] In these gas chromatographic-electroantennographic detection (GC-EAD) analyses, an insect's antenna serves as a bio-detector to help locate candidate pheromone components in complex samples.[26] Moreover, in many insects, pheromone biosynthetic pathways and receptor sites are well understood,[15,27–29] and structural similarities of pheromones among congeners have expedited pheromone identifications.[30,31] In contrast, the chemical ecology of animals lacking antennae has hardly been studied, in part, because electrophysiological techniques were not applicable.[8,17,32]

Here, we applied *non*-targeted metabolomics (XCMS online) – together with GC-MS, HPLC-MS, and behavioural bioassays – to locate and identify both contact and mate-attractant pheromone components of a web building spider. XCMS online is a free and user-friendly metabolomics software (Scripps Research CA, USA)[33] that enables analyses of data collected during mass spectrometric analyses such as GC-MS or HPLC-MS. Unlike *targeted* metabolomics, *non-targeted* metabolomics considers *all* detected ions and enables quantitative comparison of ions between samples. The software provides graphs and tables of ions as well as their relative abundance and retention times. This comprehensive approach reduces the probability of erroneously excluding peaks from analyses that are masked by other compounds (Fig. 4.1b) or occur at trace quantities (Fig. 4.1c).

Spiders have received little attention in chemical ecology research.[8] There are some 50,000 spider species but only 15 sex pheromones have been identified to date,[8,17,32] possibly because spiders lack antennae as pheromone bio-detectors (see above), and the search for pheromone receptors has met with limited success.[20,34–36] To assess how *non-untargeted* metabolomics can aid in spider pheromone research, we selected the triangulate cobweb spider, *Steatoda triangulosa*, a synanthropic, tiny (3.5–5 mm long), cosmopolitan spider inhabiting buildings.[37,38] We selected *S. triangulosa* because it belongs to a group of widow spiders (Latrodectinae) for which several

pheromones have been identified,[23,39–41] anticipating that *non-targeted* metabolomics would help us find similar pheromone components in *S. triangulosa.*

Within the Latrodectinae, females deposit contact pheromone components on their webs that elicit courtship by males upon contact. During courtship, males cut and bundle up sections of the female's web, adding their own silk in the process.[42] Contact sex pheromone components have been identified for females of the redback spider, *Latrodectus hasselti* [*N*-3-methyl-butyryl-*O*-(S)-2-methylbutyryl-L-serine methyl ester (**1**)],[23] the western black widow spider, *L. hesperus* [*N*-3-methylbutanoyl-*O*-isobutyroyl-L-serine methyl ester (**2**)],[39] the brown widow, *L. geometricus* [*N*-3-methyl-butyryl-*O*-propionyl-L-serine-methyl ester (**3**)],[41] and the false black widow spider, *Steatoda grossa* [*N*-4-methylvaleroyl-*O*-butyroyl-L-serine (**4**), *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**5**) and *N*-4-methylvaleroyl-*O*-hexanoyl-L-serine (**6**)][40] (Fig. 4.2a). In *S. grossa,* contact pheromone components – web pH-dependently – hydrolyse at the ester bond and give rise to airborne mate-attractant pheromone components: butyric acid (**7**), isobutyric acid (**8**), and hexanoic acid (**9**) (Fig. 2a).[40] *N*-4-Methylvaleroyl-L-serine (**10**), as another hydrolysis breakdown product, accumulates on webs and has no pheromonal activity[40].

In this study, we applied four analytical tools – *non-targeted* metabolomics (XCMS online), HPLC-MS, GC-MS, and behavioural bioassays – to identify the contact and mate-attractant pheromone components of *S. triangulosa.* We demonstrate that these tools in combination, but not on their own, provided decisive analytical capability to unravel the complete pheromonal communication system of *S. triangulosa*.

4.3 Methods

4.3.1 Spider rearing and web collection

Steatoda triangulosa spiders used in experiments were the F1 and F3 offspring of females collected at the Black Widow Winery [Penticton, British Columbia (B.C.), Canada, 49.5467°N, 119.5698°W]. The spiders were reared in the insectary of the Burnaby campus of Simon Fraser University at 22 °C under a reversed light cycle (12:12 h). Spiderlings were kept individually in petri dishes (100 × 20 mm)[43] fitted with

moist cotton, and fed *Drosophilia melanogaster* vinegar flies once per week. Virgin females and naïve adult males were randomly chosen for web-building and bioassays, respectively. Each female built her web for 7 days on a triangular prism (10 × 10 × 10 cm) of bamboo skewers (Goodcook, USA).[42] Webs were collected using a methanol-cleaned glass rod and were extracted overnight in 25 µL/web of acetonitrile (ACN, 99%, Sigma-Aldrich, USA). All behavioural bioassays were run during the reversed scotophase (0900 to 1700).

4.3.2 Analyses of web extracts by high-performance liquid chromatography - mass spectrometry (HPLC-MS)

Aliquots (2 µL) of web extracts of adult and subadult female *S. triangulosa* were analysed, and compared, using coupled high-performance liquid chromatography - mass spectrometry (HPLC/MS). The Bruker maXis Impact Quadrupole Time-of-Flight LC/MS system consisted of an Agilent 1200 LC fitted with a Spursil C_{18} column (30 mm × 3.0 mm, 3µm; Dikma Technologies, Foothill Ranch, CA, USA) and a Bruker maXis Impact Ultra-High Resolution tandem TOF (UHR-Qq-TOF) mass spectrometer. The LC/MS was operated with positive electrospray ionisation (+ESI) at a gas temperature of 200 ºC and a flow of 9L/min. The nebuliser was set to 4 bar and the capillary voltage to 4200 V. The column was eluted with a 0.4 mL/min flow of a solvent gradient, starting with 80% water and 20% acetonitrile, and ending with 100% acetonitrile after 4 min. The solvent system contained 0.1% formic acid to improve the peak shape of compounds.

4.3.3 Analyses of web extracts by gas chromatography - mass spectrometry (GC-MS)

Web extracts of adult virgin females were also analysed by coupled gas chromatography - mass spectrometry (GC-MS), using an Agilent 7890B GC fitted with a DB-5 GC-MS column (30 m × 0.25 mm ID, film thickness 0.25 µm) and coupled to a 5977 A MSD. The injector port of the GC was set to 250 ºC, the transfer line to 280 °C, the MS source to 230 ºC, and the MS quadrupole to 150 ºC. Helium was used as the carrier gas at a flow rate of 35 cms^{-1}. The following temperature program was used: 50 ºC held for 5 min, a 10 ºC min^{-1} increase to 280 ºC (held for 10 min). Compounds were identified by comparing their mass spectra and retention indices with those of authentic

standards that were synthesised in our laboratory. To improve chromatography of potential acids in web extracts, acids were silyl-ester derivatised[44] prior to analyses.

4.3.4 Analyses of web extracts by XCMS online

In search of further pheromone components, LC-MS analyses of web extracts of 10 adult females and 10 subadult female *S. triangulosa* were compared by XCMS online (fall 2021). The following parameters were used for the pairwise comparison: bw = 5, ppm = 10, peak width = c(2, 20), mzwidth = 0.01, and mzdiff = 0.01. Detected masses were sorted by “fold change” between the two groups and an arbitrary fold change of greater 35× was selected as the threshold.

4.3.5 Behavioural testing of contact pheromone components – T-rod bioassays

4.3.5.1 General experimental design

The ability of web extract and of specific candidate contact pheromone components to induce courtship by male *S. triangulosa* was tested in T-rod bioassays, drawing on an established protocol.[40,43,45] The T-rod apparatus consisted of a horizontal beam (8 × 0.4 cm) and a vertical beam (8 × 0.4 cm) held together by labelling tape (3 × 1.9 cm, Fisher Scientific, Ottawa, ON, CA). A piece of filter paper (2 cm^2) was attached to each distal end of the horizontal beam. The vertical beam of T-rods was inserted into plasticine (Craftsmart, Irving, TX, USA) placed in a tray (45 × 35 × 2.5 cm) partially filled with water to prevent spider males from escaping.

For each bioassay, three web equivalents in ACN, or synthetic candidate pheromone components in ACN at three web-equivalents, were applied to the randomly assigned treatment filter paper, whereas ACN was applied to the control filter paper. ACN was allowed to evaporate for 1 min before the onset of a 15-min bioassay. A randomly selected naïve male spider was then placed at the base of the vertical beam, and the time he spent courting on each filter paper was recorded. In response to the presence of female-produced or synthetic pheromone on a filter paper, the male engaged in courtship, pulling silk with his hindlegs from his spinnerets and adding it to the paper. Sensing contact pheromone, the male essentially behaves as if he were courting on the

web of a female. Replicates of experiments as part of specific research objectives were run in parallel to eliminate day effects on responses of spiders. Treatment and control arms were alternated between replicates. T-rods and filter paper were discarded after use.

4.3.5.2 Specific experiments

The effect of web extract and of specific candidate contact pheromone components on courtship behaviour by male *S. triangulosa* was tested in three sets of T-rod bioassays. In set 1 (summer 2019), parallel experiments 1 and 2 (n = 20 each) tested web extract (three web equivalents in ACN) *versus* an ACN control (Exp. 1), and synthetic candidate contact pheromone component **5** *versus* an ACN control (Exp. 2). Synthetic **5** was tested at the same amount (387 ng) as present in three web extract equivalents. In set 2 (summer 2022), parallel experiments 3 and 4 (n = 20 each) tested web extract (three web equivalents in ACN) *versus* an ACN control (Exp. 3), and a blend of synthetic candidate contact pheromone components **5** (239 ng), **11** (8 ng) and **12** (11 ng) *versus* an ACN control. In set 3 (summer 2022), parallel experiments 5–8 tested **5**, **11** and **12** in ternary combination (Exp. 5; lure composition as in Exp. 4; total lure dose: 259 ng) and singly (Exps. 6–8), each at 259 ng.

4.3.6 Chemical inferences and calculations of mate-attractant pheromone components

Drawing on previous findings that the contact pheromone components of *S. grossa* hydrolyse at the ester bond and give rise to acid mate-attractant pheromone components (Fig. 4.2b),[40] we predicted that the contact pheromone components **5**, **11** and **12** of *S. triangulosa* would also hydrolyse and release butyric acid (**7**) and isobutyric acid (**8**) as mate-attractant pheromone components. However, we could not detect **7** and **8** in GC-MS analysis of silyl-ester derivatised web extract and needed to estimate **7** and **8** based on amounts of the amide breakdown products **10** (*N*-4-methylvaleroyl-L-serine) and **13** (*N*-3-methylbutanoyl-L-serine) that originate from the hydrolysis and remain on webs (Fig. 4.2). With **10** quantified at 272 ng (1.34 mol) per web, and anticipating equal stoichiometric amounts of **8** and **10**, we decided to bioassay **8** at 124 ng per web. Moreover, with **13** not quantifiable in web extracts, we inferred the amount of **7** (5 ng)

based on the 4% of **12** (which gives rise to **7**) in the 3-component contact pheromone blend.

4.3.7 Sources of synthetic chemicals

Butyric acid (**7**) and isobutyric acid (**8**) (both 99% chemically pure) were purchased from Sigma-Aldrich (USA), and *N*-4-methylvaleroyl-*O*-isobutyryl-L-serine (**5**), *N*-3-methyl-butyryl-*O*-propionyl-L-serine (**11**), and *N*-3-methyl-butyryl-*O*-butyroyl-L-serine (**12**) were synthesised in our laboratory following the established synthesis of acylated serine derivates.[23,39,40]

4.3.8 Behavioural testing of mate-attractant pheromone components – olfactometer bioassays

Attraction of males to webs of mature females spiders and to synthetic candidate mate-attractant pheromone components **7** (butyric acid) and **8** (isobutyric acid) was tested in still-air dual-choice olfactometers (winter 2022).[46] Large Plexiglass arenas (180 cm × 12 cm × 13 cm, Fig. 3h)[46] lined with printer plot recorder paper (180 × 13 cm, Agilent, Santa-Clara, CA, USA) served as olfactometers. Two wooden prisms bearing (*i*) a female web or no web (Exp. 9, n = 30), or (*ii*) artificial (Halloween) web (45.05 ± 0.4 mg)[47] treated with synthetic **7** (5 ng) and **8** (124 ng) (Sigma-Aldrich) in ACN (75 µL), or an ACN control (75 µL) (Exp. 10, n = 30), were placed at opposite ends of the arena. For each bioassay, a single naïve male spider was placed into the centre of the arena centre and allowed 30 min to approach and contact a prism, a behavioural response recorded as first choice. After each bioassay, the paper lining, webbing, and prisms were discarded. Treatment and control sides were alternated between replicates, and the same number of replicates was run for each of two experiments to eliminate potential day effects. Each male was tested only once.

4.3.9 Statistical analysis

We analysed data using R (v. 4.3.1) and R-studio (v. 2303.06.0). A Wilcoxon Rank Sum test was used to compare the amount of time male *S. triangulosa* spent courting on filter paper treated with web extract (Exps. 1, 3) or a synthetic pheromone

blend (Exps. 2, 4). A Kruskal-Wallis Rank Sum test with Benjamini-Hochberg correction was used to compare the amount of time male *S. triangulosa* spent courting on filter paper treated with a ternary pheromone blend or single components (Exps. 4–8). A one-sided[48] binomial test[40] with Benjamini-Hochberg correction for multiple testing was used to test effects of spider webs (Exp. 9), or a binary blend of synthetic mate-attractant pheromone components (Exp. 10), on attraction of male *S. triangulosa*.

4.4 Results

4.4.1 Analyses of web extracts by HPLC-MS, GC-MS and XCMS online

HPLC-MS analyses of web extract revealed a candidate contact pheromone component with fragment ions 296.1542 (M+Na), 274.1725 (M+1) and 186.1179, matching the fragment ions and retention times of synthetic co-eluting **4** (*N*-4-methylvaleroyl-*O*-butyroyl-L-serine) and **5** (*N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine).[37] Coelution of **4** and **5** made it difficult to determine which compound was present but subsequent GC-MS analysis of esterified web extract determined that the compound was **5**.

XCMS online confirmed the presence of **5** in web extracts (Fig. 4.3b). As female spiders progressed from subadults to adults, compound **5** ions 186.1179 and 274.1725 in web extracts increased 560-fold and 341-fold, respectively. Another unknown compound (**X**), with retention time 4.42 min and fragment ion 260.1559, increased 37-fold (Fig. 4.3b).

GC-MS analysis of esterified extract in selected ion monitoring mode, searching for the 331.1815 ion of unknown **X**, revealed two isomers with retention time 21.05 min and 21.52 min. These isomers were identified as the trimethylsilyl-derivatives of *N*-3-methylbutanoyl-*O*-isobutyroyl-L-serine (**11**) and *N*-3-methylbutanoyl-*O*-butyroyl-L-serine (**12**).

4.4.2 Behavioural testing of contact pheromone components – T-rod bioassays

Web extract of adult females elicited more sustained courtship behaviour than synthetic **5** as a single contact pheromone component (W = 272, p = 0.026; Fig. 4.3e, Exps. 1, 2), indicating the presence of additional contact pheromone components in web extracts. However, the ternary blend of contact pheromone components **5**, **10** and **11** was as effective as web extract in eliciting courtship behaviour by males (W = 215.5, p = 0.665; Fig. 4.3f, Exps. 3, 4), indicating that all essential components were present in the synthetic blend. The duration of male courtship on filter paper treated with **5**, **10** and **11** as a ternary blend, and singly, differed ($\chi^2 = 14.52$, df = 3, $p < 0.001$, Fig. 4.3g, Exps. 5–8). Statistically (but not numerically), **5** was as effective as the ternary blend, and more effective than **12** but not than **11**, in prompting and sustaining courtship by males (Fig. 4.3g).

4.4.3 Behavioural testing of mate-attractant pheromone components – olfactometer bioassays

In arena olfactometers (Fig. 4.3h), prisms bearing webs of a female spider attracted more males than empty prisms (19 *vs* 9, N = 30, p = 0.043; Fig. 4.3i, Exp. 9), indicating the dissemination of airborne mate-attractant pheromone components from webs. Similarly, prisms bearing artificial (Halloween) web treated with synthetic mate-attractant pheromone components **7** and **8** attracted more males than prisms bearing Halloween web treated with a corresponding solvent control (17 *vs* 7, N = 30, p = 0.043; Fig. 4.3i, Exp. 10), indicating that **7** and **8** are the essential, and likely the only, mate-attractant pheromone components of *S. triangulosa*.

4.5 Discussion

Our study provides proof of concept that a comprehensive analytical approach, entailing non-untargeted metabolomics (XCMS online), HPLC-MS, GC-MS, and behavioural bioassays, was effective for unravelling the sex pheromone of the web-building spider *S. triangulosa*. Whereas contact pheromone component **5** (*N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine) would have been detected by conventional HPLC-MS, contact pheromone components **11** (*N*-3-methyl-butyryl-*O*-propionyl-L-

serine) and **12** (*N*-3-methyl-butyryl-*O*-butyroyl-L-serine) were discovered only through the combined application of XCMS online, GC-MS, and behavioural bioassays. Through XCMS, we found that the fragment ion 260.1559 of an unknown compound (**X**) was 37-fold more abundant in web extracts of adult females than in web extracts of subadult females. GC-MS analyses of esterified extract, selectively scanning for the indicative ion of **X**, then revealed that **X** consisted of two isomers: **11** and **12**. In T-rod behavioural bioassays with male *S. triangulosa*, a ternary blend of synthetic **5**, **11**, and **12** was as effective as adult female web extract in eliciting courtship by males, indicating that all essential contact pheromone components were present in the synthetic blend.

Metabolomics has become a routine analytical tool to screen samples for the presence or relative abundance of compounds in 'case' samples relative to reference (control) samples.[17,33] Non-targeted metabolomics has been applied e.g. in studies of diet and health,[49] sport and exercise,[50] host-microbiota,[51] drug discovery,[52] plant metabololisms,[53] organismal responses to environmental toxicants,[54] and biomarker discovery in disease diagnosis.[55]

Non-targeted metabolomics as a pheromone research tool seems to have been used in only a recent single study.[56] Liu et al.[56] hypothesised that the uropygial gland of ducks secretes chemicals that mediate sexual communication. Using LC-MS and principal component analyses, the authors found numerous metabolites in gland secretions, and noticed a gender-bias in metabolite secretions. Five compounds were significantly more abundant in secretions of males than females: picolinic acid, 3-hydroxypicolinic acid, indolacetaldehyde, 3-hydroxymethyl-glutaric acid, and 3-methyl-2-oxovaleric acid. However, whether any of these chemicals has a pheromonal signal function has yet to be tested, which may prove challenging considering the avian study organism. In our *S. triangulosa* study, we applied non-targeted metabolomics, together with mass spectrometry and behavioural bioassays, to unravel new contact pheromone components and prove their pheromonal signal function.

Prior pheromone chemistry knowledge of theridiid widow spiders (Fig. 2a)[23,39–41] aided the identification of the *S. triangulosa* sex pheromone. *Steatoda triangulosa* and *S.*

grossa share *N*-4-methylvaleroyl-*O*-isobutroyl-L-serine (**5**) as a contact pheromone component, but **5** is a minor component in *S. grossa* and the major component in *S. triangulosa* (Fig. 3). The two minor components of *S. triangulosa* – *N*-3-methyl-butyryl-*O*-propionyl-L-serine (**11**) and *N*-3-methyl-butyryl-*O*-butyroyl-L-serine (**12**) – are reported here for the first time as spider pheromone components. Notably, all currently known contact pheromone components of *Latrodectus* and *Steatoda* are acylated serine derivates with a conserved *N*-amide-*O*-ester core. Whereas pheromone components of female *Latrodectus* spp. have a methyl ester functionality and an *N*-4-methylvaleroyl-L-serine amide (**10**) rest, pheromone components of *S. grossa* and *S. triangulosa* have a free carboxylic acid – instead of a methyl ester – and either a **10** amide rest or a *N*-3-methyl-butyryl-L-serine amide (**13**) rest (Fig. 2). All data combined reveal astounding structural similarity between theridiid pheromones and imply a shared biosynthetic pathway. However, despite their common *N*-amide-*O*-ester serine motif, *Latrodectus* and *Steatoda* pheromones have unique characteristics that support the taxonomic assignment of these spiders to different genera.[37,57–59] While it has long been known that insect congeners produce structurally related pheromones, as shown in *Lymantria* moths[60–66] and *Dendroctonus* bark beetle,[67–70] our study reveals an analogous phenomenon in two genera of web-building widow spiders.

In *S. grossa*, the contact pheromone components **4**, **5** and **6** – web pH-dependently – hydrolyze at the ester bond, giving rise to the airborne mate-attractant pheromone components butyric acid (**7**), isobutyric acid (**8**) and hexanoic acid (**9**) (Fig. 2).[40] We have shown that this hydrolysis is likely catalysed by a web-borne carboxyl ester hydrolase,[40] but did not search for this type of enzyme on webs of *S. triangulosa*, anticipating a similar pheromone breakdown mechanism. We inferred that the hydrolysis of both **5** (*N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine) and **11** (*N*-3-methylbutanoyl-*O*-isobutyroyl-L-serine) would release isobutyric acid and that the hydrolysis of **12** (*N*-3-methylbutanoyl-*O*-butyroyl-L-serine) would release butyric acid as mate-attractant pheromone components (Fig. 2b). This inference proved correct because a binary blend of synthetic **7** and **8** attracted *S. triangulosa* males in arena olfactometers as effectively as female webs (Fig. 3i).

The integration of insect antennae as pheromone bio-detectors in GC-EAD analyses of complex odour samples[25,71] has expedited insect pheromone identifications and enabled the discovery of trace pheromone components.[72,73] In contrast, pheromone research in animal taxa that lack antennae, such as spiders, or that rely on olfactory epithelia in nasal cavities and/or on vomeronasal organs for odour reception, such as mammals, reptiles and amphibians,[6] has progressed at a much slower pace.[8] Pheromones are known for only 15 spiders,[17,40] and for relatively few mammals,[74,75] reptiles,[76–78] and amphibians.[78,79] This paucity of progress may be attributed to the fact that olfactory receptors are not known for spiders,[36,80,81] or are deemed fully functional for vertebrates only *in-vitro*, which would require challenging preparations for pheromone research. However, there is now emerging evidence that metabolomics may be able – to some extent – to assume the functional role of olfactory bio-detectors in pheromone research. Non-targeted metabolomics was successfully applied in sex pheromone research with ducks[56] and spiders[this study], both groups lacking antennae or equivalently effective bio-detectors for pheromone tracing. As a result, there is incentive now to apply non-targeted metabolomics for pheromone research in marine and terrestrial mammals, fish, birds, reptiles and amphibians, or even in signaling studies within and among plants.

In conclusion, we have applied non-targeted metabolomics, in combination with HPLC-MS, GC-MS, and behavioural bioassays, to unravel the sex pheromone of *S. triangulosa*. In this proof-of-concept study, we demonstrate that these four tools in combination, but not on their own, provide the analytical resolution to unravel the complete pheromonal communication system of a web-building spider. Our study provides impetus to take a similar analytical approach for pheromone research in other taxa that lack antennae or have odour receptors deemed fully functional only *in-vivo*.

4.6 Acknowledgements

We thank the Lancaster family of the Black Widow Winery for allowing us to collect spiders, and Sharon Oliver for some word processing. A.F. was supported by Graduate Fellowships from SFU, the H.R. McCarthy Bursary, and an Alexander Graham Bell Scholarship from the Natural Sciences and Engineering Research Council of Canada

(NSERC). The project was funded by an NSERC - Industrial Research Chair to G.G., with Scotts Canada Ltd. and BASF Canada Inc. as the industrial sponsors. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

4.7 References

1. Clutton-Brock, T. (2007). Sexual selection in males and females. Science *318*, 1882–1885. 10.1080/09540121.2012.701726.
2. Darwin, C. (1871). The Descent of Man and Selection in Relation to Sex (Random House, Modern Library).
3. Breithaupt, T., and Thiel, M. (2011). Chemical communication in Crustaceans (Springer Science+Business Media).
4. Karlson, P., and Lüscher, M. (1959). 'Pheromones': a new term for a class of biologically active substances. Nature *183*, 55–56. 10.1038/183055a0.
5. Wyatt, T.D. (2017). Primer pheromones. Current Biology *27*, 739–743. 10.1016/j.cub.2017.06.039.
6. Wyatt, T.D. (2014). Pheromones and Animal Behavior (Cambridge University Press) 10.1242/jeb.00738.
7. Ayasse, M., Paxton, R.J., and Tengö, J. (2001). Mating behavior and chemical communication in the order hymenoptera. Annual Review of Ecology, Evolution, and Systematics *46*, 31–78. 10.1063/1.1420510.
8. Symonds, M.R.E., and Elgar, M.A. (2008). The evolution of pheromone diversity. Trends in Ecology and Evolution *23*, 220–228. 10.1016/j.tree.2007.11.009.
9. Francke, W., and Schulz, S. (2010). Pheromones of Terrestrial Invertebrates. In Comprehensive Natural Products II, L. Mander and H.-W. Liu, eds., pp. 153–223.
10. Ayasse, M., Stökl, J., and Francke, W. (2011). Chemical ecology and pollinator-driven speciation in sexually deceptive orchids. Phytochemistry *72*, 1667–1677. 10.1016/j.phytochem.2011.03.023.
11. Littlewood, P.M.H. (1988). The chemosensory behaviour of *Lithobius forficatus* (Myriapoda: Chilopoda). 2. Bioassay and chemistry of the coxal pheromone. Journal of Zoology *215*, 523–535. 10.1111/j.1469-7998.1988.tb02857.x.
12. Beauche, F., and Richard, F.-J. (2013). The best timing of mate search in *Armadillidium vulgare* (Isopoda, Oniscidea). PloS ONE *7*, e57737. 10.1371/journal.pone.0057737.
13. Scott, A.M., Zhang, Z., Jia, L., Li, K., Zhang, Q., Dexheimer, T., Ellsworth, E., Ren, J., Chung-Davidson, Y.W., Zu, Y., et al. (2019). Spermine in semen of male sea lamprey acts as a sex pheromone. PLoS Biology *17*, 1–24. 10.1371/journal.pbio.3000332.
14. Varner, E., Gries, R., Takács, S., Fan, S., and Gries, G. (2019). Identification and field testing of volatile components in the sex attractant pheromone blend of female house mice. Journal of Chemical Ecology *45*, 18–27. 10.1007/s10886-018-1032-3.

15. Vosshall, L.B., Amrein, H., Morozov, P.S., Rzhetsky, A., and Axel, R. (1999). A spatial map of olfactory receptor expression in the *Drosophila* antenna. Cell *96*, 725–736. 10.1016/S0092-8674(00)80582-6.
16. Caskey, J. l., Watson, G. M., and Bauer, R. T. (2009). Studies on contact sex pheromones of the caridean shrimp Palaemonetes pugio: II. The role of glucosamine in mate recognition. Invertebrate Reproduction and Development *53*, 105–116. 10.1080/07924259.2009.9652296.
17. Fischer, A. (2019). Chemical communication in spiders - a methodological review. Journal of Arachnology *47*, 1–27. 10.1636/0161-8202-47.1.1.
18. Harris, D.C. (2016). Quantitative Chemical Analysis (W. H. Freeman and Company).
19. Bruice, P.Y. (2016). Organic Chemistry (Pearson).
20. Fischer, A., Schulz, S., Ayasse, M., and Uhl, G. (2021). Pheromone communication among sexes of the garden cross spider *Araneus diadematus*. The Science of Nature *108*, 1–11. 10.1007/s00114-021-01747-9.
21. Sramkova, A., Schulz, C., Twele, R., Francke, W., and Ayasse, M. (2008). Fertility signals in the bumblebee *Bombus terrestris* (Hymenoptera: Apidae). Naturwissenschaften *95*, 515–522. 10.1007/s00114-008-0353-4.
22. Gillard, J., Frenkel, J., Devos, V., Sabbe, K., Paul, C., Rempt, M., Inzé, D., Pohnert, G., Vuylsteke, M., and Vyverman, W. (2013). Metabolomics enables the structure elucidation of a diatom sex pheromone. Angewandte Chemie - International Edition *52*, 854–857. 10.1002/anie.201208175.
23. Jerhot, E., Stoltz, J.A., Andrade, M.C.B., and Schulz, S. (2010). Acylated serine derivatives: A unique class of arthropod pheromones of the Australian Redback spider, *Latrodectus hasselti*. Angewandte Chemie *49*, 2037–2040. 10.1002/anie.200906312.
24. Moorhouse, J.E., Yeadon, R., Beevor, P.S., and Nesbitt, B.F. (1969). Method for use in studies of insect chemical communication. Nature *223*, 1174–1175.
25. Arn, H., Städler, E., and Rauscher, S. (1975). The electroantennographic detector—a selective and sensitive tool in the gas chromatographic analysis of insect pheromones. Z. Naturforsch *30c*, 722–725.
26. Ma, T., Xiao, Q., Yu, Y.G., Wang, C., Zhu, C.Q., Sun, Z.H., Chen, X.Y., and Wen, X.J. (2016). Analysis of tea geometrid (*Ectropis grisescens*) pheromone gland extracts using GC-EAD and GC×GC/TOFMS. Journal of Agricultural and Food Chemistry *64*, 3161–3166. 10.1021/acs.jafc.6b00339.
27. Martin, J.P., Beyerlein, A., Dacks, A.M., Reisenman, C.E., Riffell, J.A., Lei, H., and Hildebrand, J.G. (2011). The neurobiology of insect olfaction: Sensory processing in a comparative context. Progress in Neurobiology *95*, 427–447. 10.1016/j.pneurobio.2011.09.007.
28. Blomquist, G.J., and Vogt, R.G. eds. (2003). Insect Pheromone Biochemistry and Molecular Biology (Academic Press).
29. Ando, T., Inomata, S., and Yamamoto, M. (2004). Lepidopteran sex pheromones. Topics in Current Chemistry *239*, 51–96. 10.1007/b95449.
30. Holman, L., Lanfear, R., and D'Ettorre, P. (2013). The evolution of queen pheromones in the ant genus *Lasius*. Journal of Evolutionary Biology *26*, 1549–1558. 10.1111/jeb.12162.

31. Löfstedt, C., and Kozlov, M. (1997). A phylogenetic analysis of pheromone communication in primitive moths. In Insect Pheromone Research: New Directions, R. T. Cardé and A. K. Minks, eds. (Springer US), pp. 473–489. 10.1007/978-1-4615-6371-6_41.
32. Schulz, S. (2013). Spider pheromones - a structural perspective. Journal of Chemical Ecology *39*, 1–14. 10.1007/s10886-012-0231-6.
33. Tautenhahn, R., Patti, G.J., Rinehart, D., and Siuzdak, G. (2012). XCMS online: A web-based platform to process untargeted metabolomic data. Analytical Chemistry *84*, 5035–5039. 10.1021/ac300698c.
34. Xiao, Y.-H., Zhang, J.-X., and Li, S.-Q. (2010). Male-specific (Z)-9-tricosene stimulates female mating behaviour in the spider *Pholcus beijingensis*. Proceedings of the Royal Society B *277*, 3009–3018. 10.1098/rspb.2010.0763.
35. Tichy, H., Gingl, E., Ehn, R., Papke, M., and Schulz, S. (2001). Female sex pheromone of a wandering spider (*Cupiennius salei*): Identification and sensory reception. Journal of Comparative Physiology - A *187*, 75–78. 10.1007/s003590000175.
36. Müller, C.H.G., Ganske, A.S., and Uhl, G. (2020). Ultrastructure of chemosensory tarsal tip-pore sensilla of *Argiope* spp. Audouin, 1826 (Chelicerata: Araneae: Araneidae). Journal of Morphology *281*, 1634–1659. 10.1002/jmor.21276.
37. Bradley, R.A. (2012). Common Spiders of North America 1st Edition, ed. (University of California Press).
38. Bellmann, H. (2010). Der Kosmos-Spinnenführer (Kosmos).
39. Scott, C., McCann, S., Gries, R., Khaskin, G., and Gries, G. (2015). *N*-3-Methylbutanoyl-*O*-methylpropanoyl-L-serine methyl ester – Pheromone component of western black widow females. Journal of Chemical Ecology *41*, 465–472. 10.1007/s10886-015-0582-x.
40. Fischer, A., Gries, R., Alamsetti, S.K., Hung, E., Roman Torres, A.C., Fernando, Y., Meraj, S., Ren, W., Britton, R., and Gries, G. (2022). Origin, structure and functional transition of sex pheromone components in a false widow spider. Communications Biology *5*, 1156. 10.1038/s42003-022-04072-7.
41. Baruffaldi, L. (2016). Function and diversity of sex pheromones in representative species of the black widow spiders (genus *Latrodectus*, Araneae: Theridiidae).
42. Fischer, A., Goh, X.H., Varney, J.-L.S., Blake, A.J., Takács, S., and Gries, G. (2020). Multimodal and multifunctional signaling? – Web reduction courtship behavior in a North American population of the false black widow spider. PloS ONE *15*, e0228988. 10.1371/journal.pone.0228988.
43. Fischer, A., Lee, Y., Stewart, J., and Gries, G. (2018). Dodging sexual conflict? – Sub-adult females of a web-building spider stay cryptic to mate-seeking adult males. Ethology *124*, 838–843. 10.1111/eth.12807.
44. Stalling, D., L., C.W.G., and Zumwalt, R.W. (1968). A new silylation reagent for amino acids bis (trimethylsilyl) trifluoroacetamide (BSTFA). Biochemical and Biophysical Research Communications *31*, 616–622.
45. Scott, C., Gerak, C., McCann, S., and Gries, G. (2018). The role of silk in courtship and chemical communication of the false widow spider, *Steatoda*

grossa (Araneae: Theridiidae). Journal of Ethology *36*, 191–197. 10.1007/s10164-017-0539-3.
46. Gries, R., Britton, R., Holmes, M., Zhai, H., Draper, J., and Gries, G. (2015). Bed bug aggregation pheromone finally identified. Angewandte Chemie International Edition *54*, 1135–1138. 10.1002/anie.201409890.
47. Fischer, A., Hung, E., and Gries, G. (2019). Female false black widow spiders, *Steatoda grossa*, recognize webs based on physical and chemical cues. Entomologia Experimentalis et Applicata *167*, 803–810. 10.1111/eea.12825.
48. Ruxton, G.D., and Neuhäuser, M. (2010). When should we use one-tailed hypothesis testing? Methods in Ecology and Evolution *1*, 114–117. 10.1111/j.2041-210x.2010.00014.x.
49. Zhang, X., Li, Q., Xu, Z., and Dou, J. (2020). Mass spectrometry-based metabolomics in health and medical science: a systematic review. RSC Advances. *10*, 3092–3104. 10.1039/C9RA08985C.
50. Heaney, L.M., Deighton, K., and Suzuki, T. (2019). Non-targeted metabolomics in sport and exercise science. Journal of Sports Sciences *37*, 959–967. 10.1080/02640414.2017.1305122.
51. Zhao, X., Jiang, L., Fang, X., Guo, Z., Wang, X., Shi, B., and Meng, Q. (2022). Host-microbiota interaction-mediated resistance to inflammatory bowel disease in pigs. Microbiome *10*, 115. 10.1186/s40168-022-01303-1.
52. Alarcon-Barrera, J.C., Kostidis, S., Ondo-Mendez, A., and Giera, M. (2022). Recent advances in metabolomics analysis for early drug development. Drug Discovery Today *27*, 1763–1773. 10.1016/j.drudis.2022.02.018.
53. Anzano, A., Bonanomi, G., Mazzoleni, S., and Lanzotti, V. (2022). Plant metabolomics in biotic and abiotic stress: a critical overview. Phytochemistry Reviews *21*, 503–524. 10.1007/s11101-021-09786-w.
54. Liu, L., Wu, Q., Miao, X., Fan, T., Meng, Z., Chen, X., and Zhu, W. (2022). Study on toxicity effects of environmental pollutants based on metabolomics: A review. Chemosphere *286*, 131815. 10.1016/j.chemosphere.2021.131815.
55. Baima, G., Laderosa, G., Citterio, F., Grossi, S., Romano, F., Berta, G.N., Buduneli, N., and Aimetti, M. (2021). Salivary metabolomics for the diagnosis of periodontal diseases: a systematic review with methodological quality assessment. Metabolomics *17*, 1. 10.1007/s11306-020-01754-3.
56. Liu, H., Yang, Z., He, Y., Yang, Q., Tang, Q., Yang, Z., Qi, J., Hu, Q., Bai, L., and Li, L. (2022). Metabolic profiling reveals that the olfactory cues in the duck uropygial gland potentially act as sex pheromones. Animals *12*, 413. 10.3390/ani12040413.
57. Liu, J., May-Collado, L.J., Pekár, S., and Agnarsson, I. (2016). A revised and dated phylogeny of cobweb spiders (Araneae, Araneoidea, Theridiidae): A predatory cretaceous lineage diversifying in the era of the ants (Hymenoptera, Formicidae). Molecular Phylogenetics and Evolution *94*, 658–675. 10.1016/j.ympev.2015.09.023.
58. Garb, J.E., and Hayashi, C.Y. (2013). Molecular evolution of α-latrotoxin, the exceptionally potent vertebrate neurotoxin in black widow spider venom. Molecular Biology and Evolution *30*, 999–1014. 10.1093/molbev/mst011.

59. Wheeler, W.C., Coddington, J.A., Crowley, L.M., Dimitrov, D., Goloboff, P.A., Griswold, C.E., Hormiga, G., Prendini, L., Ramírez, M.J., Sierwald, P., et al. (2017). The spider tree of life: phylogeny of Araneae based on target-gene analyses from an extensive taxon sampling. Cladistics *33*, 574–616. 10.1111/cla.12182.
60. Gries, G., Gries, R., Khaskin, G., Slessor, K.N., Grant, G.G., Liška, J., and Kapitola, P. (1996). Specificity of nun and gypsy moth sexual communication through multiple-component pheromone blends. Naturwissenschaften *83*, 382-385.
61. Schaefer, P.W., Gries, G., Gries, R., and Holden, D. (1999). Pheromone components and diel periodicity of pheromonal communication in *Lymantria fumida*. Journal of Chemical Ecology *25*, 2305–2312.
62. Gries, G., Schaefer, P.W., Khaskin, G., Hahn, R., Gries, R., and Chao, J.-T. (1999). Sex pheromone components of casuarina moth, *Lymantria xylina*. Journal of Chemical Ecology *25*, 2535–2545.
63. Gries, G., Schaefer, P.W., Gries, R., Liška, J., and Gotoh, T. (2001). Reproductive character displacement in *Lymantria monacha* from Northern Japan? Journal of Chemical Ecology *26*, 1163–1176.
64. Gries, G., Schaefer, P.W., Gries, R., Fan, Y.-B., Higashuira, Y., and Tanaka, B. (2002). 2-Methyl-(Z)-7-octadecene: Sex pheromone of allopatric *Lymantria lucescens* and *L. serva*. Journal of Chemical Ecology *28*, 469–478.
65. Gries, R., Khaskin, G., Gotoh, T., Schaefer, P.W., and Gries, G. (2005). (7*Z*,9*E*)-2-Methyl-7,9-octadecadiene: A sex pheromone component of *Lymantria bantaizana*. Journal of Chemical Ecology *31*, 879–891. 10.1007/s10886-005-3550-z.
66. Gries, R., Schaefer, P.W., Hahn, R., Khaskin, G., Ramaseshiah, G., Singh, B., Hehar, G.K., and Gries, G. (2007). Sex pheromone components of Indian Gypsy Moth, *Lymantria obfuscata*. Journal of Chemical Ecology *33*, 1774–1786. 10.1007/s10886-007-9328-8.
67. Symonds, M.R.E., and Elgar, M.A. (2004). The mode of pheromone evolution: evidence from bark beetles. Proceedings of the Royal Society B *271*, 839–846. 10.1098/rspb.2003.2647.
68. Barkawi, L.S., Francke, W., Blomquist, G.J., and Seybold, S.J. (2003). Frontalin: De novo biosynthesis of an aggregation pheromone component by *Dendroctonus* spp. bark beetles (Coleoptera: Scolytidae). Insect Biochemistry and Molecular Biology *33*, 773–788. 10.1016/S0965-1748(03)00069-9.
69. Blomquist, G.J., Figueroa-Teran, R., Aw, M., Song, M., Gorzalski, A., Abbott, N.L., Chang, E., and Tittiger, C. (2010). Pheromone production in bark beetles. Insect Biochemistry and Molecular Biology *40*, 699–712. 10.1016/j.ibmb.2010.07.013.
70. Gitau, C.W., Bashford, R., Carnegie, A.J., and Gurr, G.M. (2013). A review of semiochemicals associated with bark beetle (Coleoptera: Curculionidae: Scolytinae) pests of coniferous trees: A focus on beetle interactions with other pests and their associates. Forest Ecology and Management *297*, 1–14. 10.1016/j.foreco.2013.02.019.

71. Roelofs, W.L. (1984). Electroantennogram assays: rapid and convenient screening procedures for pheromones. In Techniques in Pheromone Research, H. E. Hummel and T. A. Miller, eds. (Springer New York), pp. 131–159. 10.1007/978-1-4612-5220-7_5.
72. Gries, R., Alamsetti, S.K., van Herk, W.G., Catton, H.A., Meers, S., Lemke, E., and Gries, G. (2021). Limoniic acid - Major component of the sex pheromones of the click beetles *Limonius canus* and *L. californicus*. Journal of Chemical Ecology *47*, 123–133. 10.1007/s10886-020-01241-y.
73. Gries, R., Khaskin, G., Gries, G., Bennett, R.G., King, G.G.S., Morewood, P., Slessor, K.N., and Morewood, W.D. (2002). (*Z*,*Z*)-4,7-Tridecadien-(*S*)-2-yl acetate: sex pheromone of Douglas-fir cone gall midge, *Contarinia oregonensis*. Journal of Chemical Ecology *28*, 2283–2297.
74. Rasmussen, L.E.L., Lee, T.D., Roelofs, W.L., Zhang, A., and Daves, G.D. (1996). Insect pheromone in elephants. Nature *379*, 684–684. 10.1038/379684a0.
75. Varner, E., Jackson, H., Mahal, M., Takács, S., Gries, R., and Gries, G. (2020). Brown rats and house mice eavesdrop on each other's volatile sex pheromone components. Scientific Reports *10*, 17701. 10.1038/s41598-020-74820-4.
76. Shine, R., and Mason, R.T. (2012). An airborne sex pheromone in snakes. Biology Letters *8*, 183–185. 10.1098/rsbl.2011.0802.
77. Mason, R.T., and Parker, M.R. (2010). Social behavior and pheromonal communication in reptiles. Journal of Comparative Physiology A *196*, 729–749. 10.1007/s00359-010-0551-3.
78. Houck, L.D. (2009). Pheromone communication in amphibians and reptiles. Annual Review of Physiology *71*, 161–176. 10.1146/annurev.physiol.010908.163134.
79. Poth, D., Wollenberg, K.C., Vences, M., and Schulz, S. (2012). Volatile amphibian pheromones: macrolides from mantellid frogs from Madagascar. Angewandte Chemie International Edition *51*, 2187–2190. 10.1002/anie.201106592.
80. Ganske, A.-S., and Uhl, G. (2018). The sensory equipment of a spider – A morphological survey of different sensillum types in both sexes of *Argiope bruennichi* (Araneae, Araneidae). Arthropod Structure & Development *47*, 144–161. 10.1016/j.asd.2018.01.001.
81. Uhl, G. (2013). Spider olfaction: attracting, detecting, luring and avoiding. In Spider Ecophysiology, W. Nentwig, ed. (Springer), pp. 141–157. 10.1007/978-3-642-33989-9.

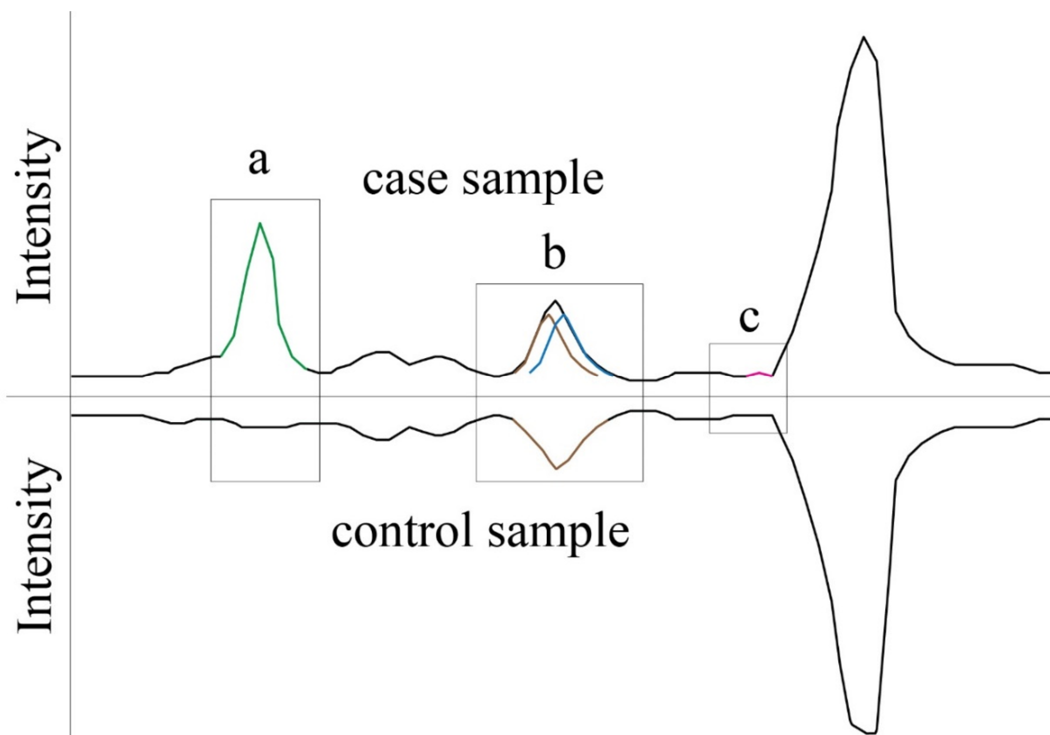


Figure 4.2: Graphical comparison of total ion chromatograms of a hypothetical case sample (upper trace) and a control sample (lower trace). (a) A unique compound (green) in the case sample is absent in the control sample. (b) A novel compound (blue) in the case sample is masked – and thus easily overlooked – by a compound (brown) present in both samples. (c) A unique trace compound (red) in the case sample might not be detected.

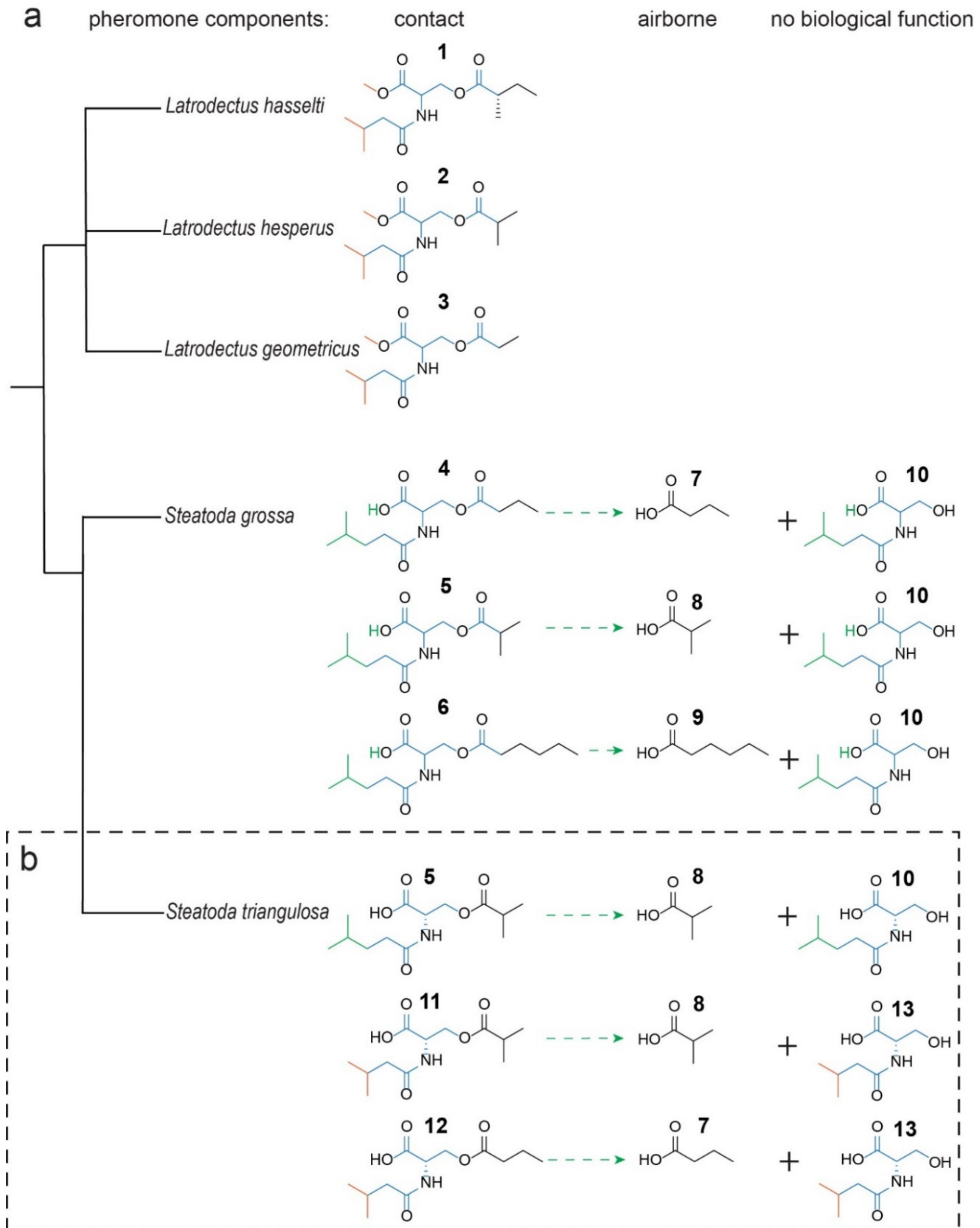


Figure 4.2: Phylogeny and comparison of pheromone components (contact & airborne) in widow spiders (Latrodectinae). (**a**) Previously known pheromone components of *Latrodectus hasselti*,[23] *L. hesperus*,[39] *L. geometricus*,[41] and *Steatoda grossa*:[40] *N*-3-methyl-butyryl-*O*-(*S*)-2-

methylbutyryl-L-serine methyl ester (**1**), *N*-3-methylbutanoyl-*O*-isobutyroyl-L-serine methyl ester (**2**), *N*-3-methyl-butyryl-*O*-propionyl-L-serine-methyl ester (**3**), *N*-4-methylvaleroyl-*O*-butyroyl-L-serine (**4**), *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**5**), and *N*-4-methylvaleroyl-*O*-hexanoyl-L-serine (**6**). The contact pheromone components **4–6** of *S. grossa* hydrolyse at the ester bond and give to three airborne mate-attractant pheromone components [butyric acid (**7**), isobutyric acid (**8**), and hexanoic acid (**9**)], whereas the amide *N*-4-methylvaleroyl-L-serine (**10**), as another hydrolysis breakdown product, remains on webs and has no behavioural activity. (**b**) Pheromone components of *Steatoda triangulosa* identified in this study. The contact pheromone components *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**5**), *N*-3-methyl-butyryl-*O*-propionyl-L-serine (**11**), and *N*-3-methyl-butyryl-*O*-butyroyl-L-serine (**12**) hydrolyse at the ester bond and give rise to two airborne mate-attractant pheromone components [butyric acid (**7**) and isobutyric acid (**8**)], whereas *N*-4-methylvaleroyl-L-serine (**10**) and *N*-3-methyl-butyryl-L-serine (**13**) accumulate on webs. Blue-coloured parts of molecules are phylogenetically conserved, whereas green-coloured parts are unique to *Steatoda*. Orange parts are shared between *Latrodectus* spp. and *S. triangulosa*.

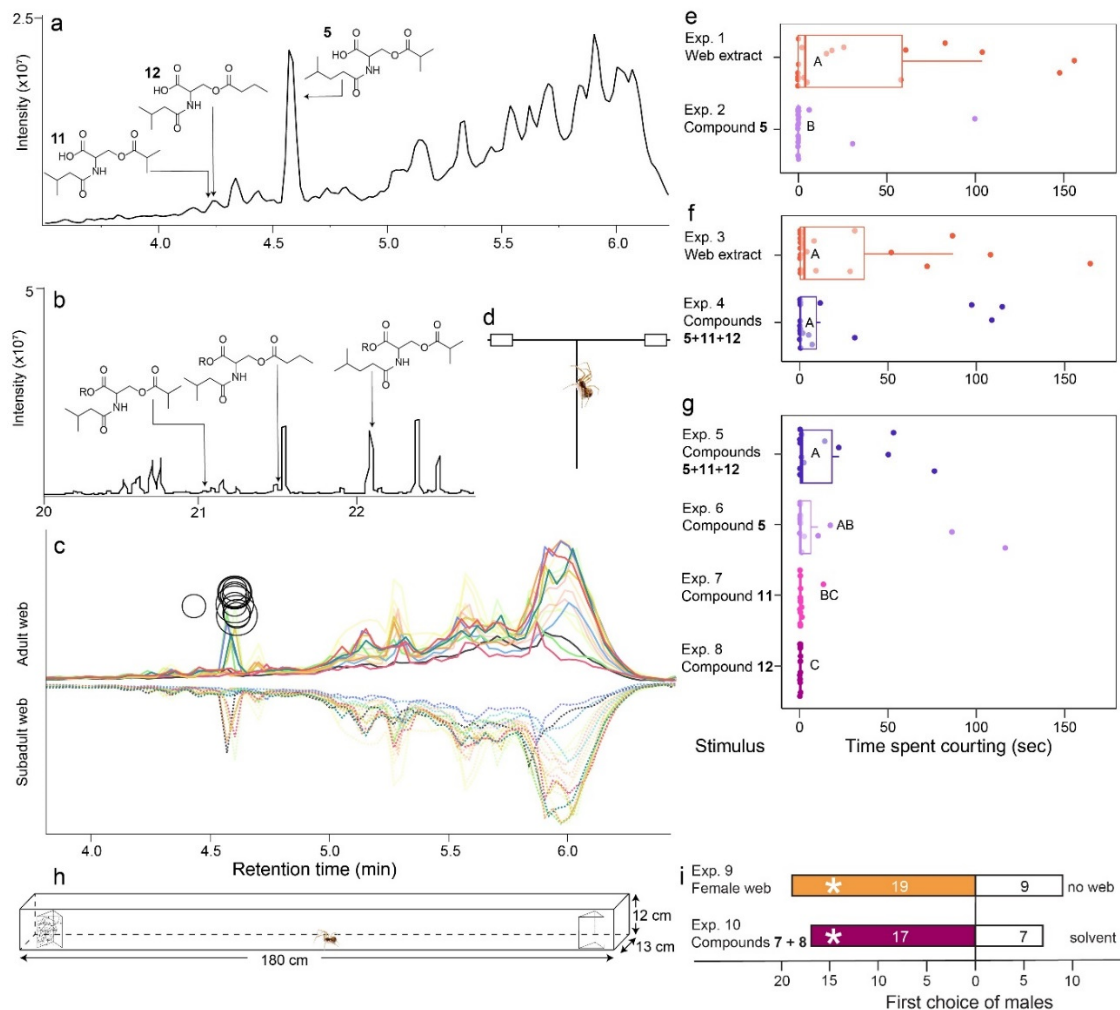


Figure 4.3: Chromatograms, experimental designs, and behavioural bioassay results. (a) Total ion chromatogram (TIC) of web extract of female *Steatoda triangulosa* analysed by high-performance liquid chromatography - mass spectrometry. (b) TIC of silyl ester-derivatised web extract of female *S. triangulosa* analysed by gas chromatography - mass spectrometry. (c) Comparative XCMS online Cloud Plots of web extracts of mature and immature female S. triangulosa (depicted by solid and dotted lines, respectively), with circles denoting a >35-fold abundance increase of fragment ions in compounds; the larger the circle, the greater the fold-change of a particular ion. (d) T-rod bioassay apparatus. (e) Effects of female *S. triangulosa* web extract (Exp. 4.1) and contact pheromone component 5 (*N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine) (Exp. 2) on courtship by *S. triangulosa* males. (f) Effects of female *S. triangulosa* web extract (Exp. 3), and a ternary blend of contact pheromone components **5**, **11** (*N*-3-methylbutanoyl-*O*-isobutyroyl-L-serine, and **12** (*N*-3-methylbutanoyl-*O*-butyroyl-L-serine) (Exp. 4), on courtship by *S. triangulosa* males. (g) Effects of contact pheromone

components **5**, **11** and **12** presented in ternary combination (Exp. 5), and singly (Exps. 6–8), on courtship by *S. triangulosa* males. (h) Arena olfactometer with prisms carrying test stimuli. (i) Attraction of male *S. triangulosa* to webs of female *S. triangulosa* (Exp. 9), and to synthetic mate-attractant pheromone components **7** (butyric acid) and **8** (isobutyric acid) in arena olfactometers. In each of subpanels e–g, different letters indicate statistical differences between test stimuli across experiments (rank sum test; $p<0.05$). In experiments 9 and 10 (subpanel i), the asterisk (*) indicates a significant preference for the test stimulus (binomial test; $p < 0.05$).

Chapter 5: Same-sex conflict in a spider – Female false black widows adjust their webs' architecture and attractiveness in response to competition for prey and mates, and to predation risk.[1]

[1]The corresponding manuscript, with Andreas Fischer, Yasasi Fernando, April Preston, Sarah Moniz-de-Sa, and Gerhard Gries as authors, is currently in peer review.

5.1 Abstract:

Female-female conflict has been neglected in animal studies. Responses of females that context-dependently compete for mates and prey, and seek safety from predators, are ideally studied with web-building spiders. Cobwebs possess unique sections for prey-capture and safety which can be quantified. We worked with *Steaoda grossa* females because their pheromone is known, and adjustments in response to mate competition could be measured. Females exposed to synthetic sex pheromone adjusted their webs, indicating perception of intra-sexual competition via their sex pheromone. When females sequentially built their webs in settings of low and high intra-sexual competition, they adjusted their webs to increase prey capture and lower predation risk. In settings with strong mate competition, females deposited more contact pheromone components on their webs and accelerated their breakdown to mate-attractant pheromone components, essentially increasing their webs' attractiveness. We show that females respond to sexual, social and natural selection pressures originating from intra-sexual conflict.

Keywords: Female-female conflict, pheromone autodetection, social selection, sexual selection, natural selection, predation risk

5.2 Introduction

Conflict is a universal phenomenon that arises from competition for resources and mates.[1] Sexual conflict originates when males and females have conflicting optimal reproductive fitness strategies, possibly leading to an evolutionary 'arms race' between males and females.[2–4] Moreover, there is conflict not only between conspecific males and females, but also among males and among females.[5–7] In this same-sex conflict, males – and rarely females – compete for mates (sexual selection[8]) or for essential resources (social selection[9]). Same-sex conflict is an evolutionary force shaping the selection of traits that increase reproductive fitness.[8] Surprisingly, female-female conflict has received little attention.[10] There has been a gender bias in selection theory, and research has prioritised males over females in studies of evolutionary selection for secondary sexual traits, such as marked coloration, large size, or striking adornments, possibly because these traits are generally more apparent in males than in females.[10] That competition among females can be an evolutionary force has only recently been acknowledged.[11]

While females rarely directly compete for mates, "maternally-biased reproductive investment renders females more likely than males to experience intense competition for resources important for reproduction".[5,6] Females competing for reproductive resources not directly associated with mates (social selection; contest competition[9,12]) may be subject to evolutionary selection for secondary sexual traits, such as larger 'weaponry', and thus improved reproductive fitness.[6] For example, female dung beetles, *Onthophagus sagittarius*, competing for animal feces as a reproductive resource are subject to selection for larger weaponry and thus improved reproductive fitness.[6] Conspecific females that are in conflict with each other over reproductive resources such as nutrients and offspring development sites must be able to sense and process information about their competitors to remain engaged in the co-evolutionary 'arms-race'.[12] Communication modalities conveying such information may be visual, vibratory (acoustic or substrate-vibration), or chemical (smell or taste) in nature.[13]

Chemical communication is thought to be the oldest mode of information transmission,[14] and sex pheromones, serving as intraspecific sexual communication signals that facilitate mate attraction, recognition, and acceptance, greatly contribute to reproductive success and survival.[15] Sex pheromones may elicit behavioural responses or cause physiological changes in receivers.[16] For example, the releaser sex pheromone of burying beetles, *Nicrophorus vespilloides*, attract mates[17], whereas the primer pheromone of queen bees, such as *Lasioglossum malachurum*, supresses ovarian development in workers.[18] Traditionally, sex pheromones were deemed to be chemical signals between females and males, and females were thought to not sense their own sex pheromone.[15,19] Consequently, female behaviour in response to female pheromones has rarely been studied.[20] However, it is now known that females of at least some insect species do sense, and respond to, their own sex pheromone.[20] For examples, females of the *cotton bollworm*, *Heliothis armigera,* the *corn earworm*, *Helicoverpa zea*, and the *Mediterranean flour moth, Ephestia kuehniella,* all avoid, or disperse from, locations with pheromone-permeated air.[21,22] However, the proximate resources for which these females compete have not been empirically studied.

Ecological theory predicts that a complex social context invokes competition for prey and mates, but little is known whether it also invokes predator defense mechanisms in prospective prey.[12,23] Aggregated animals in a complex social context are more likely than solitary animals to draw the attention of predators.[24]

Female cobweb spiders are ideal models for studying the effects of perceived same-sex competition and risk of predation.[25,26] Cobwebs, like other spider webs, have three main functions: prey capture,[27] mate attraction,[28] and safety from potential predators such as spider-hunting wasps that respond to chemical cues from spider prey.[29,30] Aggregations of cobwebs in the same micro-location (high-web-density settings)[31–34] may subject female spiders to severe competition for prey (social selection) and mates (sexual selection) as well as high predation risk (‘struggle for survival’; natural selection). Whether female spiders can sense, and respond to, their own sex pheromone, and use this ability to reduce prey and mate competition, has never been investigated.

Cobwebs, despite their seemingly unorganised appearance, have highly functional architecture to address all the spider's needs. These needs, however, are ever changing. For example, hungry spiders invest more in prey-capture silk (Fig. 5.1a) than do sated spiders.[25] Similarly, spiders in high-web-density settings with perceived competition for prey should invest heavily in silk for prey capture. Furthermore, spiders in high-web-density settings, with vast chemical cues for spider-hunting wasps to exploit, may perceive an increased risk of predation, and thus fortify their webs' safety area. Web adjustment by spiders in response to perceived competition for prey and mates, as well as risk of predation, can be measured by quantifying changes in web characteristics, such as the number of silken strands females produce for prey-capture and safety. Moreover, perceived mate competition can be assessed by quantifying the amount of courtship-inducing contact pheromone components deposited on silk, and by determining the rate of their breakdown into airborne mate-attractant pheromone components (Fig. 5.1b) [26].

In our study, we used the false black widow spider, *Steatoda grossa,* as the model species. This solitary spider commonly dwells in buildings,[35–37] where females build cobwebs with architectural characteristics resembling those of black widow webs.[38] To attract mates, *S. grossa* females deposit onto their webs three serine ester contact pheromone components (*N*-4-methylvaleroyl-*O*-butyroyl-L-serine (**1**), *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**2**) and *N*-4-methylvaleroyl-*O*-hexoyl-L-serine (**3**)) which then hydrolyse at their ester bonds, thereby releasing three corresponding mate-attractant pheromone components: butyric acid (**4**), isobutyric acid (**5**), and hexanoic acid (**6**).[26] Concurrently, the serine amide breakdown product, *N*-4-methylvaleroyl-L-serine (**7**), accumulates on the webs (Fig. 5.1a) [26]. The transition – or breakdown – of contact pheromone components to mate-attractant pheromone components is thought to be mediated by a pH-dependent enzyme, with female spiders apparently able to manipulate the breakdown rate, and thereby the attractiveness of their webs to mate-seeking males.[26]

We predicted that *S. grossa* females can sense their social context, such as their presence in low- or high-web-density settings of conspecific females, and that they adjust their web in accordance with their perceived social context. As high-web-density settings

likely come with strong same-sex competition for mates and prey, and with a high risk of predation, females would benefit from alleviating adverse effects related to competition and predation risk. Within this theoretical framework, we tested three hypotheses (H): (1) females in high-web-density settings adjust their webs to increase prey capture and lower predation risk; (2) females sense same-sex competition via airborne mate-attractant pheromone components; and (3) females in high-web-density settings increase their investment in mate attraction.

5.3 Materials and Methods

5.3.1 Spider rearing

Steatoda grossa spiders used in experiments were adult offspring of females collected on the Burnaby campus of Simon Fraser University.[40] The spiders were reared in the insectary of the Burnaby campus at 22 °C at a reversed 12L:12D photo cycle. All containers housing spiders were fitted with a moist cotton ball to increase relative humidity. Juvenile spiders were kept in petri dishes (100 × 20 mm) and provisioned once a week with *Drosophila melanogaster* vinegar flies. Sub-adults were separated by sex and kept individually. Adult virgin females were transferred from petri dishes to 300-mL clear plastic cups (Western Family, Tigard, Oregon, USA) and provisioned once a week with black blow flies, *Phormia regina*. Naïve adult virgin females were randomly chosen for experiments.

5.3.2 General bioassay procedures

5.3.2.1: Web-building for web-density measurements

Each female was placed on a triangular frame (18 × 18 × 18 × 25 cm) of bamboo skewers (Bradshaw International Inc., CA, USA) and allowed 48 h to build her web. Individual frames were set in water-filled trays to prevent the spiders from escaping. Webs to be used in experiments 1 and 2 (n = 16 each) were built in a room (3.4 × 3 × 3.2 m) at 22 °C under a reversed 12L:12D photo cycle,[40] whereas experiment 3 (n = 16) was run in four rooms (2.4 × 4.6 × 4 m; 2.4 × 1.7 × 3.2 m; 2.4 × 2 × 3.2 m; 2.4 × 3.3 × 2 m).

5.3.2.2: Web measurements

Web-measurements were taken with a thin metal rod marked in 1-cm intervals[39] by recording the number of silken strands touching the rod in each interval. Nine measurements were taken for each web (Fig. 5.1f). For the first three measurements, the rod was placed vertically 1 cm away from the vertex of the triangular prism in the retreat corner (h_R) and the non-retreat corners (h_1, h_2) of the web. The next three measurements were taken by placing the rod horizontally at the top of the retreat corner (s_R) and the non-retreat corners (s_1 and s_2) of the triangular prism, pointing to the center of the respective hypothenuses. The final three measurements were taken by placing the rod at the halfway point of the lateral edges (g_R, g_1 and g_2) to the center of the respective hypothenuse at the same height. Twenty-two counts were taken for the vertical measurements and 15 counts for the horizontal measurements from each corner of the frame. A value of 1 was added to one count for each of the nine measurements to avoid multiplication with zero in the calculations.

Investment in the safety section was calculated by multiplying the mean of vertical measurements from the retreat corner s_R by the mean of top horizontal measurements from the retreat corner h_R (Fig. 5.1f). Prey-capture investment was quantified by multiplying the mean of the mean half-way horizontal measurements from the retreat and the non-retreat corners G by the mean of the mean vertical counts from the retreat and the non-retreat corners H (Fig. 5.1f). The overall silk density was assessed as the product of the means of mean top horizontal measurements S, mean half-way down horizontal measurements G, and mean vertical measurements H (Fig. 5.1f).

5.3.3 H1: Females in high-web-density settings adjust their webs to increase prey capture and lower predation risk

5.3.3.1 Experiment 1: Web adjustments by spiders in response to sequential exposure to low- and high-web-density settings (Spring 2019)

Three virgin females were first exposed to a low-web-density setting (three web-building female spiders in the same room (3.4 × 3 × 3.2 m)) and allowed 48 h to build

their webs on frames. Thereafter, these females were removed from the frames, and web density measurements were taken. Following a 12-day intermission, the same three females were placed in a high-web-density setting (30 web-building females in the same room) and allowed 48 h to build their webs (Fig. 5.1c). These three females were then removed from the frames, and web density measurements were taken. Silk was collected from each frame with a glass rod (0.5 cm × 17.5 cm) and extracted in methanol for 24 h[41] for chemical analysis.

5.3.3.2 Experiment 2: Web adjustments by spiders in response to sequential exposure to high- and low-web-density settings (Spring 2019)

To control for potentially confounding sequential exposure effects, the order of exposure was reversed in experiment 2. Three naïve females were first exposed to a high-web-density setting (30 web-building females in the same room (3.4 × 3 × 3.2 m)), and after a 12-day intermission, were exposed to the low-web-density setting, consisting of the three test spiders in a room (Fig. 5.1d). Webs were obtained, measured, and extracted as previously described.

5.3.4 H2: Females sense same-sex competition via airborne mate-attractant pheromone components.

5.3.4.1 Experiment 3: Web adjustments by spiders in response to sequential exposure to a low-web-density setting and to synthetic pheromone at a concentration mimicking a high-web-density setting (Summer 2019)

To test whether females detect the numerical web-density of conspecific females based on mate-attractant pheromone components, we modified the design of experiment 1. We used synthetic pheromone, instead of 30 female spiders, to purport a high-web-density setting. Three naïve virgin females were first exposed to the low-web-density setting, and were subsequently exposed to the same low-density setting but permeated with synthetic pheromone (**4**, **5**, **6**) at a concentration equivalent to a high-web-density setting.[26] Synthetic mate-attracting pheromone components were released from 27 400-μL Eppendorf vials, each containing **4** (0.112 μg), **5** (2.8 μg), and **6** (1.52 μg) dissolved

in 200 µL of mineral oil. Any effects of the mineral oil were controlled by adding 27 Eppendorf vials containing plain mineral oil to the low-web-density setting (Fig. 5.1e). Each Eppendorf vial was perforated with a single hole using a No. 3 insect pin.

5.3.5 H3: Females in high-web-density settings increase their investment in mate attraction.

Increased investment in mate attraction was measured by quantifying the amount of contact pheromone components females deposit on their web, and by calculating the rate of contact pheromone component breakdown into sex attractant pheromone components.

5.3.5.1 Quantification of contact pheromone components (Summer 2019)

Potential adjustments in the amount of contact sex pheromone components deposited by females on their webs in response to sequential exposure to (*i*) low- and high-web-density settings or *vice versa* (Exps. 1 & 2), or (*ii*) to a low-web-density setting followed by exposure to synthetic pheromone at a concentration equivalent to a high-web-density setting (Exp. 3), were analysed following established procedures.[26] Briefly, each web measured in experiments 1–3 was removed from its frame and then extracted for 24 h in methanol (50 µL, 99.9% HPLC grade, Fisher Chemical, Ottawa, Canada). To avoid clogging of the analytical instruments, web extracts were pre-purified using a Waters 600 high performance liquid chromatograph (HPLC, Waters Corporation, Milford, MA, USA; 600 Controller, 2487 Dual Absorbance Detector, Delta 600 pump) fitted with a Synergy Hydro Reverse Phase C18 column (250 mm × 4.6 mm, 4 microns; Phenomenex, Torrance, CA, USA) which was eluted with isocratic acetonitrile (99.9% HPLC grade, Fisher Chemical, Ottawa, Canada) at 1 mL/min. The pheromone-containing fraction (3.00 - 4.4 min) was collected and concentrated to 1 mL. For pheromone quantification, aliquots (2 µL) were injected into a Bruker maXis Impact Quadrupole Time-of-Flight LC/MS System comprising an Agilent 1200 HPLC and a Bruker maXis Impact Ultra-High Resolution tandem TOF (UHR-Qq-TOF) mass spectrometer. The Agilent HPLC was fitted with a spursil C18 column (30 mm × 3.0 mm, 3 microns; Dikma Technologies, Foothill Ranch, CA, USA) which was heated to 30 °C and eluted

with a solvent gradient (0.4 mL/min), starting with 80% water and 20% acetonitrile, and ending – after 4 min – with 100% acetonitrile. The solvent system contained 0.1% formic acid to enhance the peak shape of compounds. The mass spectrometer was set to positive electrospray ionization (+ESI) with a gas temperature of 200 °C and a gas flow of 9 L/min. The nebulizer was set to 4 bar and the capillary voltage to 4200 V. The major pheromone component **1** (ion 296, M+Na) was selected as a representative pheromone component. A calibration curve was established using synthetic **1** at 5 ng/µL, 2.5 ng/µL, 0.5 ng/µL, 0.25 ng/µL and 0.05 ng/µL.

5.3.5.2 Experiment 4: Calculation of breakdown rate of contact pheromone components to mate-attractant pheromone components (Spring 2022)

Three naïve virgin females (n = 15) were first exposed to a low-web-density setting, and then to the same low-web-density setting but permeated with synthetic pheromone at a concentration equivalent to a high-web-density setting. The webs of the three spiders per replicate were pooled, extracted in acetonitrile, and analysed by HPLC-MS without prior purification. Contact pheromone components **1**, **2**, and **3** as well as the breakdown product **7** were quantified, and the breakdown ratio was calculated by dividing **7** by the sum of the **7** + **1** + **2** + **3** (Fig. 5.1b).

5.3.6 Statistical analyses

Data (Appendix Table 3.2) were analysed statistically using R.[42] Data on web architectural elements, as well as amounts of contact pheromone components deposited on webs, were analysed for effects of social context using a repeated measures design. Webs from each group of three spiders was measured twice (N = 16 each, Exps. 1–3). We used generalised linear mixed models with tweedie family function of the glmmTMB package[43] to account for repeated measures of spiders within each group. A Type III ANOVA of the 'car' package (Anova) was used to test for significance of social-context effects (low- or high-web-density setting) on response variables.[44] Model assumptions were checked using the DHARMa package.[45] Breakdown rates of contact pheromone components to mate-attractant pheromone components in relation to social context were

quantified using a linear mixed effects model to account for the repeated measures of each group (Exp. 4).

5.4 Results

5.4.1 H1: Females in high-web-density settings adjust their webs to increase prey capture and lower predation risk.

5.4.1.1 Experiment 1: Web adjustments by spiders in response to sequential exposure to low- and high-web-density settings

Females sequentially exposed to low- and high-web-density settings of conspecific females almost doubled their prey capture- and safety-related silk investments (prey-capture: $\chi^2 = 14.29$, df = 1, $p < 0.001$; safety: $\chi^2 = 15.93$, df = 1, $p < 0.001$; Exp. 1, Fig. 5.2a+b). The overall web density tripled with the transition to a more competitive social setting ($\chi^2 = 23.41$, df = 1, $p < 0.001$, Exp. 1, Fig. 5.2c). Across all groups, three spiders were lost between exposures and were excluded from data analyses.

5.4.1.2 Experiment 2: Web adjustments by spiders in response to sequential exposure to high- and low-web-density settings

Females sequentially exposed to high- and low-web-density settings of conspecific females decreased their prey capture- and safety-related silk investments by ~ 40% (prey-capture: $\chi^2 = 20.53$, df = 1, $p < 0.001$; safety: $\chi^2 = 13.62$, df = 1, $p < 0.001$; Exp. 2, Fig. 5.2d+e). The overall silk investment decreased by almost half with the transition to a less competitive setting ($\chi^2 = 12.85$, df = 1, $p < 0.001$; Exp. 2, Fig. 5.2f). Across all groups, three spiders were lost between exposures and were excluded from data analyses.

5.4.2 Hypothesis 2: Females sense same-sex competition via airborne mate-attractant pheromone components.

5.4.2.1 Experiment 3: Web adjustments by spiders in response to sequential exposure to a low-web-density setting and to synthetic pheromone at a concentration mimicking a high-web-density setting.

Females sequentially exposed to a low-web-density setting and to synthetic sex attractant pheromone components at a dose equivalent to that of a high-web-density setting adjusted their web as if they were exposed to a high-web-density setting, indicating recognition of social context based on the presence and concentration of airborne pheromone components. Silk investment in prey-capture increased by 85% (χ^2 = 26.10, df = 1, p < 0.001; Exp. 3, Fig. 5.3a), while silk investment in safety increased by 185% (χ^2 = 100.15, df = 1, p < 0.001; Exp. 5.3, Fig. 5.3b). The overall web-density almost quadrupled (χ^2 = 82.67, df = 1, p < 0.001, Exp. 3, Fig. 5.3c). Five spiders were lost between exposures and were excluded from data analysis.

5.4.3 H3: Females in high-web-density settings increase their investment in mate attraction.

Females sequentially exposed to low- and high-web-density settings increased (49%) the amount of contact pheromone components they deposited on their webs (χ^2 = 16.44, df = 1, p < 0.001; Exp. 1, Fig. 5.4a). Conversely, females sequentially exposed to high- and low-web-density settings decreased (57%) the amount of contact pheromone components they deposited on their webs (χ^2 = 33.87, df = 1, p < 0.001; Exp. 2, Fig. 5.4b). Females sequentially exposed to a low-web-density setting and to synthetic mate attractant pheromone components at a dose equivalent to that of a high-web-density setting (*i*) increased (69%) the amount of contact sex pheromone components they deposited on their webs (χ^2 = 23.79, df = 1, p < 0.001, Exp. 3, Fig. 5.4c), and (*ii*) increased (60%) the breakdown rate of contact pheromone to sex attractant pheromone components (χ^2 = 5.28, df = 1, p = 0.022; Exp. 4, Fig. 5.4d), essentially increasing their investment in mate attraction.

5.5 Discussion

Our data show that female *S. grossa* sense intra-sexual competition for prey and mates, and predation risk, via airborne mate-attractant pheromone components. In response to perceived intra-sexual competition, female spiders adjusted their webs to increase prey capture and lower predation risk and increased their investment in mate attraction.

Solitary web-building spiders can occur in large aggregations[31,34,46–49] that seem to present specific benefits to aggregation members, prompting them to remain in aggregations. For example, female western black widows, *Latrodectus hesperus,* were reluctant to leave aggregations even when their webs were severely disturbed, and unestablished spiders delayed relocation to new microhabitats when webs of conspecifics were present.[31] These data suggest that the benefits of staying together in a suitable microhabitat outweigh the costs of relocation, such as travel costs, mortality risk, and failure to find a new habitat.[50,51] The presence of established conspecifics in a microhabitat may provide social information about habitat quality and prey availability, and may help save costs and time for habitat assessment.[33,46,52] Also, relocating and rebuilding a new web elsewhere is energetically costly to cobweb spiders which – unlike orb-weaving spiders – do not recycle their silk.[50,53]

Group living, however, has potential trade-offs. Spiders that have settled in groups will draw greater attention of predators – such as spider-hunting wasps – that exploit the chemical cues of their spider prey[28,29]. Conversely, there is safety in numbers in that the *per capita* predation risk may decrease due to the 3-dimensional architecture of their webs.[54] Similarly, female spider webs in aggregation will likely emanate more mate-attractant pheromone than single webs, and thus be more attractive to males.[34] However, group-living females must then compete with each other for access to these prospective mates. Finally, although webs in aggregations may collectively capture more prey, prey captures for each individual female spider may suffer.

To study how female spiders respond to social context, such as their presence in a high-web-density settings with all its trade-offs for mate and prey competition as well as

predation risk, we worked with the false black widow spider, *S. grossa*, a phylogenetic close relative of *L. hesperus*.[55] We worked with *S. grossa*, instead of *L. hesperus,* because its sex pheromone is fully characterised,[26] allowing us to assess whether and to what extent female *S. grossa* adjust their pheromonal signaling in response to perceived mate competition. With the pheromone known, we could also experimentally test whether *S. grossa* females sense their social setting via airborne mate-attractant pheromone components. The very nature of the 3-dimensional *S. grossa* web, with distinct safety and prey capture sections,[25,38] further allowed us to assess whether spiders adjust their webs to alleviate adverse effects related to competition for prey and to predation risk.

Our experimental data support the hypothesis that female *S. grossa* in high-web-density settings adjust their webs to increase prey capture and lower predation risk. We tested this hypothesis by sequentially exposing spiders to low- and high-web-density settings, and *vice versa*, allowing the same spiders to build their webs in each type of setting. Measuring the resulting web characteristics revealed that the spiders adjusted their webs in accordance with changes in social context. Progressing from low- to high-web-density settings with stronger competition for prey and predation risk, female spiders produced more silk strands for prey capture and fortified their webs' safety sections (Fig. 5.2). Conversely, progressing from high- to low-web-density settings with lower competition for prey and risk of predation, spiders curbed their silk production (Fig. 5.2), obviously saving energy. All data combined clearly indicate that the experimental spiders were aware of their social context. While it is well established that animals gauge habitat suitability, in part, by the presence or absence of conspecifics[56] it was not known that web-building spiders can sense the presence of conspecifics, and adjust their webs in relation to the perceived level of competition and threat of predation by natural enemies.

Olfaction is the sensory modality underlying the detection of intra-sexual competitors. With webs being physically well separated in our experiments (Fig. 5.1), and with these web-building spiders deemed not to have good vision,[57,58] and female spiders do not produce sounds[58], we predicted airborne chemicals (sex pheromones) to be the signals or cues revealing the presence of intra-sexual competitors. Spiders – including widow spiders – do produce sex pheromones, of which some have been

identified.[26,28,59,60] Although female-produced sex pheromones primarily serve in sexual communication to attract males,[28,61] it was conceivable that female spiders can sense their own pheromones (pheromone autodetection), and use this ability to gauge intra-sexual competition. To date, pheromone autodetection is known for a few insect species,[20] and is believed to reduce mate competition. Pheromone autodetection by spiders was suggested[60] but not experimentally tested. With the female *S. grossa* sex pheromone available in our lab, we could test whether it serves a role in intra-sexual signaling among females. To study this question, we allowed three female spiders to build their webs in a confined room, then removed their webs, and – after a 12-day intermission – allowed the same three spiders to re-build their webs in the same room but permeated with synthetic mate-attractant pheromone at a concentration indicative of a high-web-density setting. Our findings that these pheromone-exposed spiders rebuilt their webs with enhanced prey capture and safety functions support the hypothesis of pheromone autodetection by female *S. grossa*. The pheromone receptor(s), however, remain unknown.[62]

Female *S. grossa* responded to perceived mate-competition by (*i*) depositing greater amounts of contact pheromone components on their webs (Fig. 5.4 a-c), and (*ii*) accelerating their breakdown to mate-attractant pheromone components (Fig. 5.4d), essentially increasing their webs' attractiveness to mate-seeking males. The mechanisms underlying this chemical breakdown are not fully understood, but there is convincing evidence that direct saponification alone is insufficient to explain the observed breakdown rates.[26] Instead, a web-borne carboxyl ester hydrolase enzyme, which is present on webs of *S. grossa*[26] and *L. hesperus,*[63] is deemed responsible for the breakdown of contact pheromone components to mate-attractant pheromone components.[26] The concept is appealing because enzyme activity is pH-dependent,[64] and spiders can manipulate the pH of their silk.[65] This enzyme concept could be experimentally tested by altering the webs' pH and by studying the pH-dependent enzymatic pheromone breakdown. Alternatively, synthetic contact pheromone components could be exposed to synthetic enzyme in different pH milieus, and the resulting pheromone breakdown rates could be measured. Regardless of the outcome of these experiments, our data (Fig. 5.4) indicate that female *S. grossa* do manipulate their webs' attractiveness in response to perceived mate competition. These results are

intriguing because, to date, timed pheromone production and dissemination are known only in insects.[66]

Our study demonstrates that aggregations of *S. grossa* webs represent a complex social context that invoked intra-sexual conflict among female *S. grossa.* Females competed with each other for access to mates and prey, and thus were concurrently subject to sexual and social selections.[5,8,9,23] Sex pheromones are a key determinant of mate-attraction and reproductive success in many animal taxa,[15,67,68] and thus are subject to selection as a secondary sexual trait. In response to same-sex competition, female *S. grossa* disseminated more mate-attractant pheromone from their webs (Fig. 5.4d), and thus became more apparent to mate-seeking males. Concurrent resource competition among female *S. grossa* was evident in web adjustments that increased the likelihood of prey capture (Figs. 5.2a+d, 5.3a). As well-fed animals typically have greater reproductive capacity,[69] female *S. grossa* in web aggregations are under social selection pressure for reproductive resources such as access to prey.

The complex social context presented by *S. grossa* web aggregations not only invoked intra-sexual competition for prey and mates, but it also invoked predator defense responses, as indicated by web adjustments to fortify the webs' safety section (Figs. 5.2b+e, 5.3b). Generally, animals in aggregations are more likely than solitary animals to draw the attention of predators,[70] but aggregated animals reduce individual predation risk through a dilution effect such that the *per capita* risk of predation decreases with increasing group size.[70] That *S. grossa* females did not rely on the dilution effect as a predator escape mechanism, but – instead – lowered predation risk by strengthening their webs' safety section (Figs. 5.2b+e, 5.3b), indicates that *S. grossa* females in a complex social context are under significant natural selection pressure for survival. This selection pressure is likely exerted by spider-hunting wasps that respond to chemical cues from spider prey,[29] with spiders in aggregations likely being semiochemically more apparent, and thus more attractive, to predatory wasps than single spiders. This selection pressure may be enhanced by predatory birds, amphibians and other spiders,[27] that may also eavesdrop on chemical cues from aggregated spider webs.

In conclusion, our study adds to the scarce body of literature on female-female conflict in animals.[10] We show that female *S. grossa* spiders can sense their own pheromone, and use this ability to gauge social context. In a complex social context, female *S. grossa* increase their competitiveness for mates and prey by disseminating more pheromone from their webs and by enhancing their webs' prey capture function. Concurrently, they reduce predation risk by fortifying their webs' safety section. All data combined indicate that intra-sexual conflict of female *S. grossa* generates sexual, social and natural selection pressures that – in this perfect model system – could be separately studied and quantified.

5.6 Acknowledgements

We thank Jaime-Lynne Varney and Emmanuel Hung for assistance during preliminary studies, Hongwen Chen, Sharon Oliver, Hanna Watkins and Em Lim for technical advice. A.F. was supported by Graduate Fellowships from SFU, the H.R. McCarthy Bursary, and an Alexander Graham Bell Scholarship from the Natural Sciences and Engineering Research Council of Canada (NSERC). The project was funded by an NSERC - Industrial Research Chair to G.G., with Scotts Canada Ltd. and BASF Canada Inc. as the industrial sponsors. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

5.7 References

1. Davies, N. B., Krebs, J. R. & West, S. A. *An Introduction to Behavioural Ecology*. (Wiley-Blackwell, 2012).
2. Chapman, T., Arnqvist, G., Bangham, J. & Rowe, L. Sexual conflict. *Trends in Ecology & Evolution* **18**, 41–47 (2003).
3. Engel, K. C. *et al.* A hormone-related female anti-aphrodisiac signals temporary infertility and causes sexual abstinence to synchronize parental care. *Nature communications* **7**, 11035 (2016).
4. Chapman, T. Sexual conflict: mechanisms and emerging themes in resistance biology. *The American Naturalist* **192**, 217–229 (2018).
5. Clutton-Brock, T. Sexual selection in females. *Animal Behaviour* **77**, 3–11 (2009).

6. Watson, N.L., and Simmons, L.W. (2010). Reproductive competition promotes the evolution of female weaponry. Proceedings of the Royal Society B: Biological Sciences 277, 2035-2040. 10.1098/rspb.2009.2335.
7. Rico-Guevara, A. & Hurme, K. J. Intrasexually selected weapons: Intrasexually selected weapons. *Biol Rev* **94**, 60–101 (2019).
8. Darwin, C. *The Descent of Man and Selection in Relation to Sex*. (Random House, Modern Library, 1871).
9. West-Eberhard, M. J. Sexual selection, social competition, and speciation. *The Quarterly Review of Biology* **58**, 29 (1983).
10. Ah-King, M. The history of sexual selection research provides insights as to why females are still understudied. *Nat Commun* **13**, 6976 (2022).
11. Tang-Martínez, Z. Rethinking bateman's principles: challenging persistent myths of sexually reluctant females and promiscuous males. *The Journal of Sex Research* **53**, 532–559 (2016).
12. Harari, A.R., and Steinitz, H. (2013). The evolution of female sex pheromones. Current Zoology. *59*, 569-578. 10.1093/czoolo/59.4.569.
13. Gries, G. From chemical to communication ecology. *Journal of Chemical Ecology* **40**, 309 (2014).
14. Breithaupt, T. & Thiel, M. *Chemical communication in crustaceans*. *Chemical Communication in Crustaceans* (2011). doi:10.1007/978-0-387-77101-4.
15. Wyatt, T. D. *Pheromones and animal behavior*. *University of Oxford* (Cambridge University Press, 2014). doi:10.1242/jeb.00738.
16. Wyatt, T. D. Primer pheromones. *Current Biology* **27**, 739–743 (2017).
17. Chemnitz, J., Bagrii, N., Ayasse, M. & Steiger, S. Variation in sex pheromone emission does not reflect immunocompetence but affects attractiveness of male burying beetles—a combination of laboratory and field experiments. *Science of Nature* **104**, (2017).
18. Steitz, I. & Ayasse, M. Macrocyclic lactones act as a queen pheromone in a primitively eusocial sweat bee. *Current Biology* **30**, 1136–1141 (2020).
19. Steiger, S. & Stökl, J. The role of sexual selection in the evolution of chemical signals in insects. *Insects* **5**, 423–438 (2014).
20. Holdcraft, R., Rodriguez-Saona, C. & Stelinski, L. L. Pheromone autodetection: Evidence and implications. *Insects* (2016) doi:10.3390/insects7020017.
21. Saad, A. & Scott, D. Repellency of pheromones released by females of *Heliothis armigera* and *H. zea* to females of both species. *Entomologia Experimentalis et Applicata* **30**, 123–127 (1981).
22. Trematerra, P. & Battaini, F. Control of *Ephestia kuehniella* Zeller by mass-trapping. *Journal of Applied Entomology* **104**, 336–340 (1987).
23. Clutton-Brock, T. H. & Huchard, E. Social competition and selection in males and females. *Phil. Trans. R. Soc. B* **368**, 20130074 (2013).
24. Ayelo, P. M. *et al.* Exploring the kairomone-based foraging behaviour of natural enemies to enhance biological control: a review. *Front. Ecol. Evol.* **9**, 641974 (2021).
25. Blackledge, T. A. & Zevenbergen, J. M. Condition-dependent spider web architecture in the western black widow, *Latrodectus hesperus*. *Animal Behaviour* **73**, 855–864 (2007).

26. Fischer, A. *et al.* Origin, structure and functional transition of sex pheromone components in a false widow spider. *Commun Biol* **5**, 1156 (2022).
27. Foelix, R. *Biologie der Spinnen*. (Chimaira, 2015).
28. Fischer, A. Chemical communication in spiders - a methodological review. *Journal of Arachnology* **47**, 1–27 (2019).
29. Uma, D. B. & Weiss, M. R. Chemical mediation of prey recognition by spider-hunting wasps. *Ethology* **116**, 85–95 (2010).
30. Uma, D. B. & Weiss, M. R. Flee or fight: ontogenetic changes in the behavior of cobweb spiders in encounters with spider-hunting wasps. *Environmental Entomology* **41**, 1474–1480 (2012).
31. Salomon, M. Social environment and feeding state influence movement decisions in a web-building spider. *Ethology* **115**, 916–927 (2009).
32. Salomon, M., Vibert, S. & Bennett, R. G. Habitat use by western black widow spiders (*Latrodectus hesperus*) in coastal British Columbia: evidence of facultative group living. *Canadian Journal of Zoology* **88**, 334–346 (2010).
33. Fischer, A., Hung, E. & Gries, G. Female false black widow spiders, *Steatoda grossa*, recognize webs based on physical and chemical cues. *Entomologia Experimentalis et Applicata* **167**, 803–810 (2019).
34. Scott, C. E., McCann, S. & Andrade, M. C. B. Black widows as plastic wallflowers: female choosiness increases with indicators of high mate availability in a natural population. *Scientific Reports* **10**, 8981 (2020).
35. Bellmann, H. *Der Kosmos-Spinnenführer*. (Kosmos, 2010).
36. Bradley, R. A. *Common Spiders of North America*. (University of California Press, 2012).
37. Welzenberger, R. Paarungsverhalten und Fortpflanzungssystem der kosmopolitischen Kugelspinne *Steatoda grossa* (Araneae , Theridiidae). (Leopold-Franzens-Universität Innsbruck, 2018).
38. Benjamin, S. P. & Zschokke, S. Webs of theridiid spiders: construction, structure and evolution. *Biological Journal of the Linnean Society* **78**, 293–305 (2003).
39. Rypstra, A. L. Building a better insect trap; An experimental investigation of prey capture in a variety of spider webs. *Oecologia* (1982) doi:10.1007/BF00349008.
40. Fischer, A., Lee, Y., Stewart, J., and Gries, G. (2018). Dodging sexual conflict?—Sub-adult females of a web-building spider stay cryptic to mate-seeking adult males. Ethology. *124*, 838-843. 10.1111/eth.12807.
41. Fischer, A., Hung, E., Amiri, N. & Gries, G. 'Mine or thine': indiscriminate responses to own and conspecific webs and egg sacs by the false black widow spider, *Steatoda grossa* (Araneae: Theridiidae). *Journal of Ethology* **38**, 241–245 (2020).
42. R Core Team. *R: A language and environment for statistical computing*. (R Foundation for Statistical Computing, 2022).
43. Brooks, M. E. *et al.* glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *The R Journal* **9**, 378–400 (2017).
44. Fox, J. *et al. An R companion to applied Regression*. (Sage, 2019).
45. Hartig, F. DHARMa: Residual diagnostics for hierarchical (multi-level / mixed) regression models. (2022).

46. Hodge, M. A. & Storfer-Isser, A. Conspecific and heterospecific attraction: A mechanism of web-site selection leading to aggregation formation by web-building spiders. *Ethology* **103**, 815–826 (1997).
47. Harwood, J. D., Sunderland, K. D. & Symondson, W. O. C. Web-location by linyphiid spiders: prey-specific aggregation and foraging strategies. *J Anim Ecology* **72**, 745–756 (2003).
48. Mougenot, F., Combe, M. & Jeanson, R. Ontogenesis and dynamics of aggregation in a solitary spider. *Animal Behaviour* **84**, 391–398 (2012).
49. Pessman, B. J., Hays, M., Agpawa, E. & Hebets, E. A. Urbanization affects web abundance and aggregation of a funnel-weaving spider, Agelenopsis pennsylvanica (Agelenidae). *Urban Ecosyst* **in press**, (2023).
50. Tanaka, K. Energetic cost of web construction and its effect on web relocation in the web-building spider *Agelena limbata*. *Oecologia* **81**, 459–464 (1989).
51. Lubin, Y., Carolina, N. & Kotzman, M. Web-relocation and habitat selection in a desert widow spider. *Ecology* **74**, 1915–1928 (1993).
52. Dall, S. R. X., Giraldeau, L.-A., Olsson, O., McNamara, J. M. & Stephens, D. W. Information and its use by animals in evolutionary ecology. *Trends in Ecology and Evolution* **20**, 187–193 (2005).
53. Janetos, A. C. Foraging tactics of two guilds of web-spinning spiders. *Behavioral Ecology and Sociobiology* **10**, 19–27 (1982).
54. Blackledge, T. A., Coddington, J. A. & Gillespie, R. G. Are three-dimensional spider webs defensive adaptations? *Ecology Letters* **6**, 13–18 (2003).
55. Wheeler, W. C. *et al.* The spider tree of life: Phylogeny of araneae based on target-gene analyses from an extensive taxon sampling. *Cladistics* **33**, 574–616 (2017).
56. Buxton, V. L., Enos, J. K., Sperry, J. H. & Ward, M. P. A review of conspecific attraction for habitat selection across taxa. *Ecology and Evolution* **10**, 12690–12699 (2020).
57. Huber, B. A. Sexual selection research on spiders: progress and biases. *Biological Reviews* **80**, 363–385 (2005).
58. Uhl, G. & Elias, D. Communication. in *Spider Behaviour* (ed. Herberstein, M. E.) 127–189 (Cambridge University Press, 2011).
59. Schulz, S. Spider pheromones - a structural perspective. *Journal of Chemical Ecology* **39**, 1–14 (2013).
60. Fischer, A., Schulz, S., Ayasse, M. & Uhl, G. Pheromone communication among sexes of the garden cross spider *Araneus diadematus*. *The Science of Nature* **108**, 1–11 (2021).
61. Gaskett, A. C. Spider sex pheromones: emission, reception, structures, and functions. *Biological Reviews* **82**, 27–48 (2007).
62. Müller, C. H. G., Ganske, A. S. & Uhl, G. Ultrastructure of chemosensory tarsal tip-pore sensilla of *Argiope* spp. Audouin, 1826 (Chelicerata: Araneae: Araneidae). *Journal of Morphology* **281**, 1634–1659 (2020).
63. Chaw, R. C., Correa-Garhwal, S. M., Clarke, T. H., Ayoub, N. A. & Hayashi, C. Y. Proteomic evidence for components of spider silk synthesis from black widow silk glands and fibers. *Journal of Proteome Research* **14**, 4223–4231 (2015).

64. Bugg, T. *Introduction to Enzyme and Coenzyme Chemistry*. *Introduction to Enzyme and Coenzyme Chemistr* (Blackwell Publishing, 2012). doi:10.1002/9781444305364.
65. Heim, M., Keerl, D. & Scheibel, T. Spider silk: From soluble protein to extraordinary fiber. *Angewandte Chemie - International Edition* **48**, 2–15 (2009).
66. Levi-Zada, A. & Byers, J. A. Circadian rhythms of insect pheromone titer, calling, emission, and response: a review. *Sci Nat* **108**, 35 (2021).
67. Ayasse, M., Paxton, R. J. & Tengö, J. Mating behavior and chemical communication in the order hymenoptera. *Annual Review of Ecology, Evolution, and Systematics* **46**, 31–78 (2001).
68. Ando, T., Inomata, S. & Yamamoto, M. Lepidopteran sex pheromones. *Topics in Current Chemistry* **239**, 51–96 (2004).
69. Šet, J. *et al.* Sex-specific developmental trajectories in an extremely sexually size dimorphic spider. *Sci Nat* **108**, 54 (2021).
70. Ioannou, C. Grouping and Predation. in *Encyclopedia of Evolutionary Psychological Science* (eds. Shackelford, T. K. & Weekes-Shackelford, V. A.) 1–6 (Springer International Publishing, 2017). doi:10.1007/978-3-319-16999-6_2699-1.

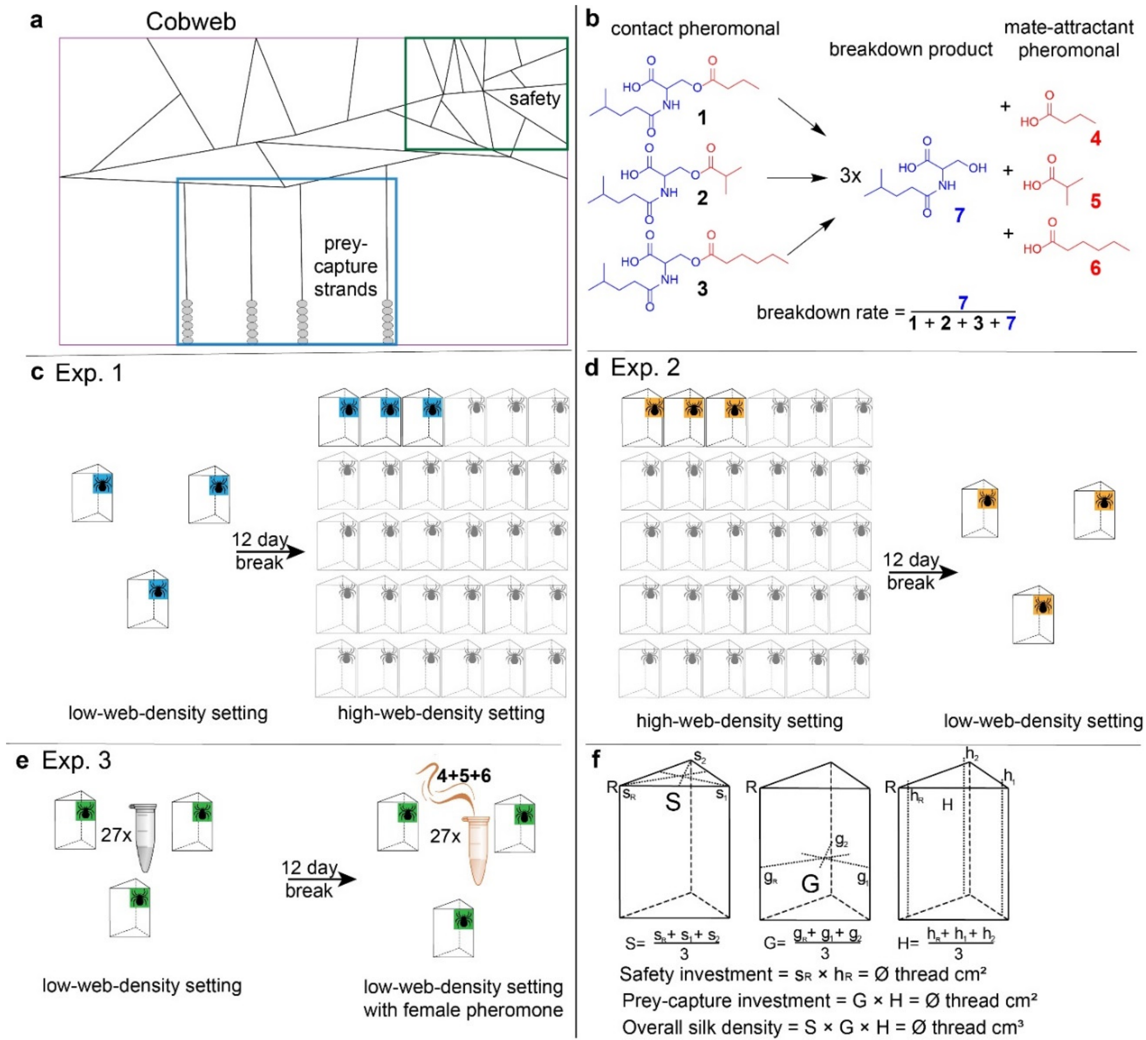


Figure 5.1: Graphical illustrations of a cobweb and experimental designs. (a) cobweb depicting the safety (retreat) section (green square) with numerous silk strands, and glue-impregnated prey-capture lines (blue square) anchored to the ground. (b) Pheromone components of female *Steatoda grossa*: three serine ester contact pheromone components [*N*-4-methylvaleroyl-*O*-butyroyl-L-serine (**1**), *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**2**) and *N*-4-methylvaleroyl-*O*-hexoyl-L-serine (**3**)] prompt courtship by males, hydrolyse at the ester bond, and give rise to three corresponding mate-attracting acid pheromone components (red) [butyric (**4**), isobutyric (**5**), hexanoic (**6**)], while the serine amide breakdown product (blue), *N*-4-methylvaleroyl-L-serine (7), remains and accumulates on the web. The rate of the hydrolysis breakdown determines the web's attractiveness to males. (c) Design of experiment 1: Three female S. grossa build their webs for 48 h on three separate 3-dimensional frames (low-web-density setting); after a 12-day intermission, the same three females built their webs together with 27 other females (high-web-density setting). (d) Design of experiment 2: three females first built their

webs in a high-web-density setting, and after a 12-day intermission, built webs in a low-web-density setting. (e) Design of experiment 3: three females built their webs first in a low-web-density setting, and after a 12-day intermission, built webs in the same low-web-density setting, but permeated with synthetic mate-attracting pheromone components (**4**, **5**, **6**) at a concentration equivalent to a high-web-density setting. Pheromone components were formulated in mineral oil and released from 27 Eppendorf vials; during the first exposure, Eppendorf vials contained only plain mineral oil. (f) Web-measurements were taken with a thin metal rod marked in 1-cm intervals[39] by recording the number of silken strands touching the rod in each interval. The rod was placed either vertically 1 cm away from the vertex of the triangular prism in the retreat corner (h_R) and the non-retreat corners (h_1, h_2) of the web, or horizontally at the top of the retreat corner (S_R) and the non-retreat corners (s_1 and s_2) of the triangular prism, pointing to the center of the respective hypothenuses Similar horizontal measurements were taken at the halfway-height point of the lateral edges.

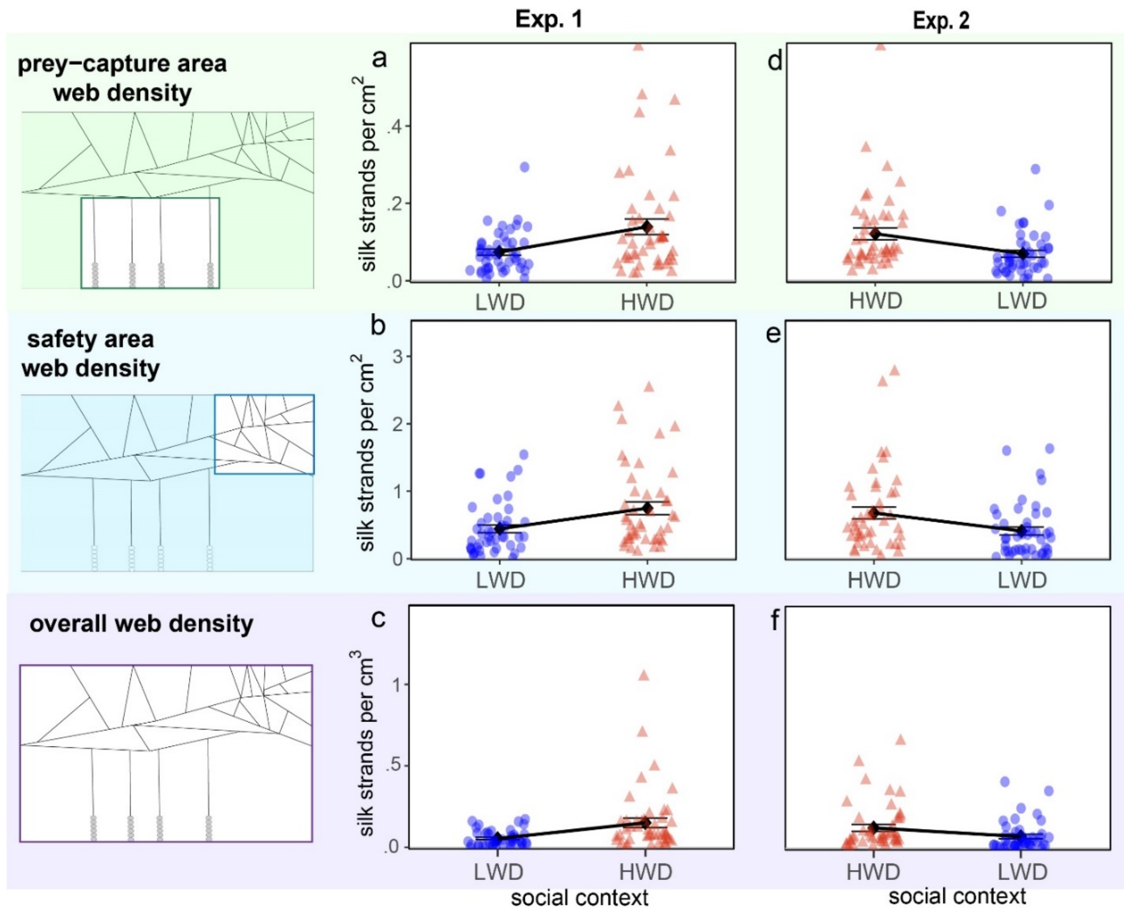


Figure 5.2: Web adjustments by female false black widow spiders in response to changes in social context. When groups of three test spiders each (n = 16) first built their webs in a low-web-density setting ('LWD'; three test spiders only), and then rebuilt their webs in a high-web-density setting ('HWD'; three test spiders together with 27 further spiders) (see Fig. 1), the groups of test spiders rebuilding their webs produced more silk strands for prey capture and safety, and overall, likely in response to perceived greater competition for prey, and predation risk. Conversely, when groups of three test spiders each (n = 16) first built their webs in a HWD setting, and then rebuilt their webs in a LWD setting, they produced fewer silk strands for prey capture and safety, and overall. Blue dots and red triangles indicate data of experimental replicates, and black squares with whiskers represent the mean and standard error. Web adjustments in each subpanel were statistically significant ($p < 0.001$; GLMM).

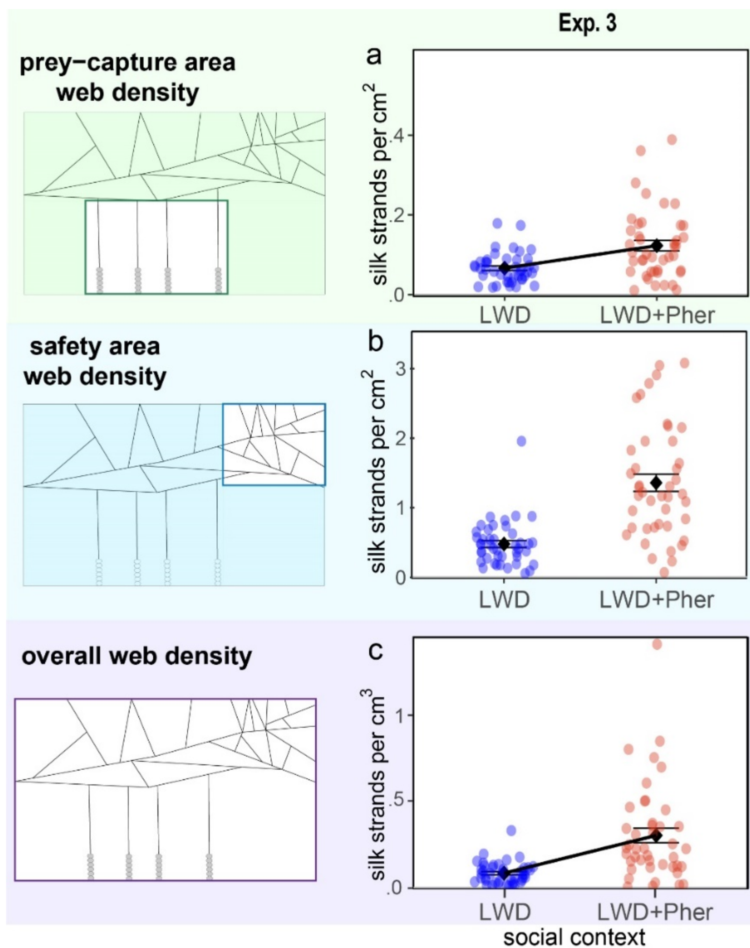


Figure 5.3: Web adjustments by female false black widow spiders in response to synthetic sex pheromone indicating social-context change. When groups of three test spiders each (n = 16) first built their webs in a low-web-density setting ('LWD'; three test spiders only), and then rebuilt their webs in a low-web-density setting while sensing synthetic pheromone at a concentration equivalent to a high-web-density setting ('LWD+Pher'), the groups of test spiders rebuilding their webs produced more silk strands for prey capture and safety, and overall, in response synthetic pheromone indicating greater competition for prey, and predation risk. Blue dots and red triangles indicate data of experimental replicates, and black squares with whiskers represent the mean and standard error. Web adjustments in each subpanel were statistically significant ($p < 0.001$; GLMM).

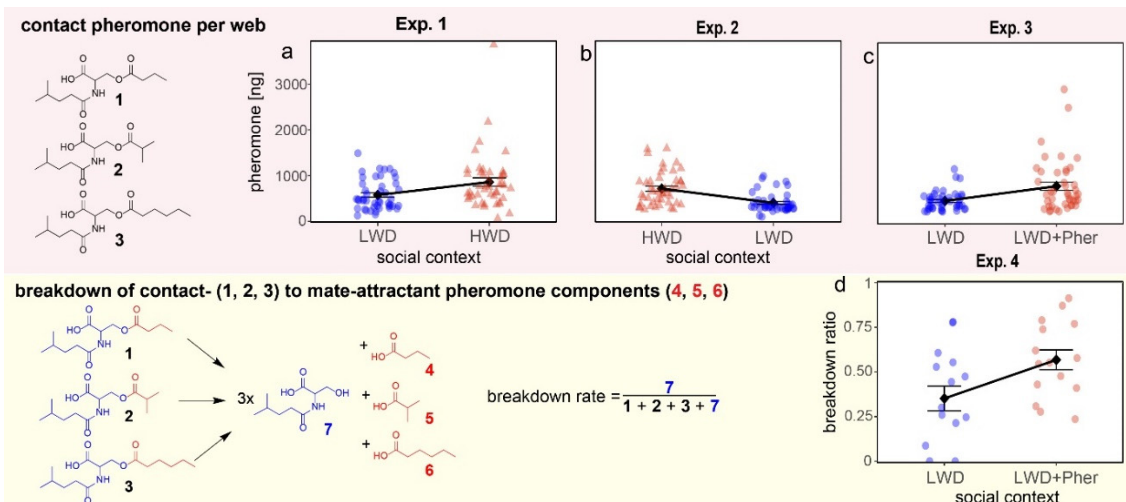


Figure 5.4: Adjustments for mate attraction by female false black widow spiders in response to perceived mate competition. When groups of three test spiders each (n = 16) first built their webs in a low-web-density setting ('LWD'; three test spiders only), and then (a+b) rebuilt their webs in a high-web-density setting ('HWD'; three test spiders together with 27 further spiders), or (c) rebuilt their webs in a low-web-density setting while sensing synthetic pheromone at a concentration equivalent to a high-web-density setting ('LWD+Pher') (see Fig. 1), the groups of test spiders rebuilding their webs deposited more contact pheromone components [*N*-4-methylvaleroyl-*O*-butyroyl-L-serine (**1**), *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**2**) and *N*-4-methylvaleroyl-*O*-hexoyl-L-serine (**3**)] on their webs (upper row) and accelerated their breakdown to mate-attractant pheromone components (red), [butyric (**4**), isobutyric (**5**), hexanoic (**6**)], essentially enhancing their webs' attractiveness to mate-seeking males. The serine amide breakdown product (blue), N-4-methylvaleroyl-L-serine (**7**), accumulates on the web. The rate of the hydrolysis breakdown determines the web's attractiveness to males. Blue dots and red triangles indicate data of experimental replicates, and black squares with whiskers represent the mean and standard error. Note changes in the amounts of contact pheromone components deposited on webs (Exps. 1-3; each $p < 0.001$, GLMM), and in the breakdown rate of contact pheromone components to mate-attractant pheromone components (Exp. 4; $p = 0.022$, GLMM) in response to perceived mate competition.

Chapter 6 Multimodal and multifunctional signaling? – Web reduction courtship behavior in a North American population of the false black widow spider[1]

[1]The corresponding manuscript has been published in *PLoS ONE* (Volume 15, e0228988, 2022), with the following authors: Andreas Fischer, Xiang Hao Goh, Jamie-Lynne S. Varney, Adam J. Blake, Stephen Takács, and Gerhard Gries

6.1 Abstract

Males of widow spiders courting on the web of females engage in web reduction behavior which entails excising a section of the web, bundling it up, and wrapping it with their silk. Males of the false black widow spider, *Steatoda grossa*, in European populations also produce stridulatory courtship sound which has not yet been studied in their invaded North American range. Working with a North American population of *S. grossa*, we tested the hypotheses that (1) web reduction by males renders webs less attractive to rival males; (2) deposition of silk by courting males has an inter-sexual (male-female) signal function that enhances their likelihood of copulation; and (3) stridulatory sound is a courtship signal of males. Testing anemotactic attraction of males in Y-tube olfactometer experiments revealed that reduced webs (indicative of a mated female) and intact webs (indicative of a virgin female) were equally attractive to males. Recording courtship behavior of males with either functional (silk-releasing) spinnerets or spinnerets experimentally occluded on the web of virgin females showed that males with functional spinnerets were more likely to copulate with the female they courted. Although males possess the stridulatory apparatus to produce courtship sound, they did not stridulate when courting or copulating on the web of females. Our data support the conclusion that web reduction behavior of *S. grossa* males in their invaded North American range has no long-range effect on mate seeking males. Instead, web reduction behavior has an inter-sexual signaling function that seems to be linked to functional spinnerets of the courting male. The signal produced by a male likely entails a volatile silk-borne pheromone but may also embody a gauge of his endurance (the amount of time he engages in web reduction causing web vibrations).

6.2 Introduction

During courtship, many animals produce multi-modal signals that function between prospective mates. These inter-sexual (male-female) signals (i) offer information about the signaler including sex, age, dominance and health [1–4], (ii) reduce aggression between partners [5], and (iii) render the female receptive to the male [6]. Courting males display diverse signals of one or more sensory modalities. Males of birds of paradise, e.g., use visual signals “showing off” their extraordinary plumage [7], male crickets stridulate producing sound [8], male tiger moths emit pheromones [9], and males of web-building spiders vibrate the female’s web [10].

Concurrently, one or both sexes may also produce intra-sexual signals that deter potential rivals [11,12]. Inter- and intra-sexual signals can be identical [11,13] or different [14–16]. For example, the boatwhistle vocalizations of male Lusitanian toadfish, *Halobatrachus didactylus*, have dual functions, serving a role during male-female courtship and as a male-male territorial signal [11].

Courtship signals may be adjusted according to the environmental setting. In the increasingly noisy urban “soundscape”, birds upshift frequency components of their songs thus improving the apparency of their signals [17,18]. Urban habitats especially can create reproductive isolation barriers and thus genetic bottlenecks in various taxa [19–22]. Sexual selection pressure modulates courtship behavior including the honesty of sexual communication signals [23,24]. Males of the Hermann’s tortoise, *Testudo hermanni hermanni*, engage in courtship that enables a female to assess their condition [25].

Courtship signals with multiple components and modalities (e.g., courting males exhibiting visual displays, emitting pheromone or sound, generating substrate-borne vibrations, all concurrently) offer rich opportunities to investigate how courted females integrate this complex information and use it to select mates [26]. Two main hypotheses have been proposed for the evolution of such multi-modal sexual signals: (1) different signals, or signal modalities, each convey different information (the ‘multiple message’ hypothesis) and (2) different signals convey the same information (the ‘backup message’

hypothesis) [26]. By experimentally suppressing one or more signal modalities of the courting male, and by studying the behavioral responses of courted females and rival males, the information content, relative importance and the intended recipient of each signal modality can be deduced.

Web-building spiders are potential model organisms to study the specific function(s) of multimodal courtship signals. For example, males of the western black widow spider, *Latrodectus hesperus*, court a female by cutting sections of her web (potential vibratory signals) and bundling them with their own silk (potential pheromonal signal) [27]. Either signal, or the combined effect of both signals, renders the female receptive and decreases the likelihood of aggression towards the male [5,27,28]. Web reduction may also reduce the attractiveness of the female's web to rival males [13]. However, the underlying mechanisms for the decreased attractiveness of reduced webs are not well understood. Bundled-up and compacted sections of a female's web have reduced surface area and thus are thought to curtail pheromone dissemination [29]. Also, the male's silk may block emanation of female pheromone from bundled web sections and/or may release a pheromone deterrent to other males [30].

Courtship signals of the closely related [31] false black widow spider, *Steatoda grossa*, seem even more complex and thus worthy of study. Males of a North American population engage in web-reduction behavior resembling that of *L. hesperus* [32–34], whereas *S. grossa* males in Europe produce audible stridulatory courtship sound (1 kHz and 3-7 kHz) [34–38] by abdominal up- and down movements (0.008 s and 0.005 s, respectively) causing teeth-like structures on the abdomen to scrape over ridges on the cephalothorax (prosoma) [38] . To produce 1 kHz, the up- and down movement of the abdomen requires 0.008 sec and 0.005 sec respectively [38] The resulting stridulatory sound is thought to have both a male-male and a male-female signal function [39,40]. Moreover, two recent studies on *S. grossa* in Europe noted both stridulatory courtship sound and web reduction behavior by males [33,34], suggesting an intricate interplay of sound, vibratory and pheromonal courtship signals conveyed by males. Whether males of *S. grossa* in North America, following the introduction of *S. grossa* to the New World early in the last century [41] also produce courtship sound has yet to be studied. Chemical

communication, in contrast, is well documented. Silk of virgin females, and methanol extract thereof, both trigger web reduction and silk deposition by males [32]. Observations that males, engaging or not in web reduction behavior during pre-copulatory courtship, deposit silk on the female's web [32], imply – but not experimentally prove – a sexual communication function of male silk or silk-borne pheromone. The male's multi-modal courtship also includes vibratory elements such as abdomen vibrations, pulling web strings with one or more appendages, front-leg drumming, and drumming on the female's 4th leg pair [32,34,38].

Courting *S. grossa* males exhibit two prominent behavioral elements: (i) web reduction (vibratory signals) with silk deposition (potential pheromonal signal), and (ii) abdomen vibrations (vibratory signal and potential auditory signal). This paper aims to study the specific functions and signaling modalities of these courtship behaviors by assessing their effects on female aggression towards males, copulatory success of males, and curtailed male competition. Working with a North American population of *S. grossa*, we tested the hypotheses that (1) web reduction by males renders webs less attractive to rival males; (2) deposition of silk by courting males has an inter-sexual signal function that enhances their likelihood of copulation; and (3) stridulatory sound is a courtship signal of males.

6.3 Methods

6.3.1 Model organism

Female *S. grossa* build their cobwebs in dry and warm places, often within buildings. Females and males live up to 6 and 1.5 years, respectively [38,42]. Males are polygynous and females are polyandrous with first sperm precedence [32,34,38], as reported in *L. hesperus*. Unlike *Latrodectus* males, mature *S. grossa* males build webs for prey-capture [38]. Female *S. grossa* have been observed to cannibalize males during copulation [33].

6.3.2 Experimental spiders

Experimental spiders were the F1 generation offspring of 182 mated females collected in hallways of Simon Fraser University [43]. Two weeks after juveniles hatched (from many different cocoons), they were separated and kept singly in Petri dishes (100 × 20 mm) and provisioned once a week with vinegar flies *Drosophila melanogaster*. Sub-adult males and sub-adult females were kept in separate rooms to prevent males from undergoing accelerated maturation [44,45]. Once a week, sub-adult and adult females and males were fed larvae of the beetle *Tenebrio molitor* and adult black blow flies, *Phormia regina*, respectively. Adult males were kept in petri dishes (100 × 200 mm), whereas adult females were kept in 300-ml clear plastic cups (Western Family, Tigard, OR, USA). All spiders had access to water in cotton wicks re-moistened once a week. Spiders were maintained at 22 °C under a reversed photoperiod (12 h:12 h). All experiments were run during the scotophase. Only mature males (>7 days post final moult) and mature virgin females (>10 days post final moult) [30] were tested in experiments. Male-female pairs in courtship trials were not siblings.

6.3.3 H1: Web reduction by males renders webs less to rival males.

Hypothesis 1 was tested in Y-tube Pyrex glass olfactometer experiments. For each experimental replicate, a male was introduced into a glass "holding" tube (2 × 26 cm) and allowed 2 min to acclimatize before the tube was connected via a glass joint to the Y-tube olfactometer (main stem: 24 cm, side arms: 21 cm, diam: 2.5 cm) [46]. A translucent oven bag (30 × 31 cm, Toppits, Mengen, Germany) containing a test stimulus such as a female web was secured to the orifice of each side arm. The opposite opening of the bag was secured to a glass tube (1.5 × 4 cm) to facilitate airflow. Bamboo skewers placed into the holding tube and the Y-tube facilitated locomotion of the bioassay male [47]. To initiate a bioassay, an air pump was connected to the holding tube, drawing air at 100 ml/min through the olfactometer. A male that entered the olfactometer within the 5-min bioassay period was classed a responder and two behavioral parameters were recorded: (a) his first choice of oven bag and (b) his engagement, or not, in web-reduction behavior within that bag. Only one of 30 identical olfactometers was deployed for a bioassay at a time and always in the same position. Following a bioassay, the bamboo skewers and bags were discarded, and the glassware was cleaned with soap water and then heated in a

drying oven at 100 °C for 3 h. In experiments 1-4, 139 males in total were bioassayed, two of which were tested in both experiments 1 and 2, and two other ones in both experiments 1 and 3.

To obtain webs as test stimuli, each randomly chosen virgin female was allowed two days to build her web on an equilateral bamboo frame (8.5 × 8.5 × 8.5 cm) residing in a tray of water [similar to 32]. Thereafter, she was lured to a web-free section of the frame by gentle tapping vibrations and then offered a bamboo stick to walk off the frame on her own accord. This procedure ensured the integrity of her newly spun web. As webs with or without the female are appealing to males (reviewed in [30]), we opted to test webs on their own.

To obtain a reduced web as a test stimulus, a male was allowed 1 h to enter a web and engage in web reduction. To obtain the wrapped-up section of a web, any visibly bundled-up section was excised from the remainder of that web. To determine whether web reduction by a male renders webs unattractive to rival males, three experiments were run in parallel. Males were given a choice between (a) a frame bearing a female web and an empty frame (Exp. 1, n = 41), (b) a frame bearing an intact female web and a frame bearing a reduced female web (see above) (Exp. 2, n = 41), and (c) a frame bearing the visibly wrapped-up section of a web and a frame bearing the remainder of that same web (Exp. 3, n = 41). Similar numbers of replicates of each experiment were run on the same day, and the position of stimuli in each experiment was randomized. To rule out experimental side bias, males were also offered a choice between two empty frames (Exp. 4, n = 20).

6.3.4 H2: Deposition of silk by courting males has an inter-sexual signal function that enhances their likelihood of copulation.

To test hypothesis 2, the spinnerets of treatment males, but not control males, were rendered non-functional (Exp. 5). Treatment males were anesthetized with CO_2 and their spinnerets were sealed with super glue gel (LePage, ON, Canada) which was applied under a dissecting microscope via the tip of a 32-gauge silver wire (Bead Landing, TX, USA). CO_2 -anesthetized control males received the same amount of super

glue gel applied to their dorsal abdomen [48]. After glue application, both treatment and control males were given at least 2 h to acclimatize and were then bioassayed within 24 h.

To control for potential effects of male and female size, and mass, on courtship and copulation success, a "condition index" was determined for each spider using regression residuals of the log-transformed body weight and size at maturation [49,50]. Prior to testing in experiments, spiders were measured alive. The weight of each male and female was measured on a calibrated scale (Denver Instrument Company TR-204, NY, USA) with an accuracy of 0.1 mg. The size of each male and female was approximated by taking photographs of the first pair of legs under a microscope (Nikon Instruments SMZ1500, NY, USA) with a built-in digital camera (Nikon Instruments DXM1200F, NY, USA), and by measuring the tibia-patella lengths with ImageJ (National Institutes of Health, USA) [50].

For each bioassay replicate (n = 20), two males closely matched in size and condition were assigned to become the treatment or the control male. The two females in each replicate were selected in the same way. The mean percentage difference in the weight and tibia-patella length between male and female pairs were all below 9%. Females were placed for two days on bamboo frames (30 × 25 × 22 cm) residing in a tray of water to build webs (as in Exps. 1-3).

Each treatment or control male was introduced to the web of a virgin female, residing in a plexiglass box (30.5 × 30.5 × 42 cm) with the female on her web. Courtship was video-recorded for 3 h with two HD cameras (Handycam HDR-XR550; Sony, Tokyo, Japan) under white fluorescent light (2 × 32-watt FO32/835/ECO T8; Sylvania, Wilmington, USA). White-light illumination was chosen to improve the image quality for analyses bearing in mind that *S. grossa* females and males do court and copulate under white light [32]. Behavioural elements like web reduction behavior, latency to copulation, copulation, female aggression (male fleeing in response to female movement) and sexual cannibalism of the male were all determined from the video recordings. As females live up to six years and produce eggs throughout their lives after having copulated once

[38,42], we did not quantify the offspring they produced because their lifetime reproductive fitness exceeded the timeframe of this study.

6.3.5 H3: Stridulatory sound is a courtship signal of males.

The stridulatory sound of *S. grossa* males in Europe is in the frequency range of 1-7 kHz [34,38]. To test for potential auditory signals produced by courting or copulating males, 20 male-female pairs were video- and sound-recorded, of which 10 pairs each were recorded with a digital sampling rate of 10 kHz and 40 kHz, respectively (Exp. 6). The higher sampling rate (Nyquist frequency) took into account that stridulatory sound of *S. grossa* may include frequency components of up to 11 kHz [34]. For each pair, the male and female were randomly selected. The plexiglass box which housed a female web was positioned in the middle of a sound-dampened room and fitted with an AKG CK 61-ULS condenser microphone (AKG Acoustics, Nashville, TN, USA). The microphone was connected to a Dell desktop computer (Dell, Round Rock, TX, USA) equipped with a 16-bit National Instruments (NI) data acquisition card (NI PCIe-6259) (DAQ) and programmed with LabVIEW 7.1 (NI, Austin, TX, USA). The signal-to-noise ratio was improved by pre-amplifying (NI SC-2040 amplifier) potential spider-produced sound prior to digitizing at 10 or 40 kHz via the DAQ card and digitizing the sound on computer [51]. Behavioral elements of males entailing abdominal movements which may produce stridulatory sound [32], namely web jerking (the male vibrating the web with his entire body) and copulation, were analyzed for sound including 30 s before they commenced and 30 s after they ended. These paired video and audio recordings were supplemented with audio recordings of background noise in the absence of spiders which were then analyzed for sound in the range of 0 -5 kHz (sampling rate of recording: 10 kHz) and 1-11 kHz (sampling rate of recording: 40 kHz) using LabVIEW's Joint Time Frequency Analyzer. To estimate the frequency (Hz) of the males' abdominal movements during courtship (sensu [32]), high speed video recordings were obtained (Exp. 7). To this end, 12 males were randomly selected and paired with one of 12 females, each on her own web. Abdominal movements of males during courtship were recorded at 30 and 960 frames per second using a Galaxy S9 camera (Samsung, Seoul, South Korea), and were analyzed frame-by-frame using VLC media player (VideoLAN, Paris, France).

Scanning electron micrographs (SEM) of the stridulatory apparatus of male *S. grossa* were obtained at the BioImaging Facility of the University of British Columbia (Vancouver, BC, Canada) using a Hitachi S-4700 instrument (Hitachi, Tokyo, Japan). After males were cold-euthanized, their abdomen and prosoma were severed and air-dried for 48 h. Both tagmata were then mounted with double-sided carbon tape on aluminum pin stubs at an angle most conducive for viewing of the stridulatory apparatus. After sputter-coating both tagmata with a 15-nm thick layer of gold using the rotary-planetary-tilting stage of a Cressington 208HR instrument at a 60-mA current, SEMs were taken using various imaging modes and accelerating voltage.

6.3.6 Statistical Analyses

Data were analyzed with R [52,53]. In experiments 1-4, first-choice responses were analyzed by χ^2-tests against an expected frequency of 50:50, whereas the proportion of males that engaged in web-reduction in response to either of the two presented test stimuli was compared with a χ^2-test. Data of experiment 5, which tested the effect of spinneret occlusion on the occurrence of specific behavior (web-reduction, copulation, cannibalism), were analyzed with either a generalized linear model (GLM) or a generalized linear mixed model (GLMM), with spinneret treatment included as the sole fixed effect. Mixed effect models [54] incorporated the effect of treatment and control male pairs into the models as a random intercept. If variance in these intercepts approached 0, mixed models were abandoned in favor of a simple χ^2-test. We also analyzed the effect of duration of web-reduction behavior on the latency to copulation and the occurrence of copulation and cannibalism by females with GLMs or GLMMs, with duration as the sole fixed effect.

6.4 Results

6.4.1 H1: Web reduction by males renders webs less attractive to rival males (Exps. 1–4)

Males significantly more often entered first those oven bags that enclosed a frame with an intact web than oven bags enclosing an empty control frame ($\chi^2 = 7.05$, df = 1, n = 41, $p < 0.001$; Fig. 6.1, Exp. 1). In contrast, oven bags enclosing a frame with an intact

web or a frame with a reduced web were entered first equally by males ($\chi^2 = 0.10$, df = 1, n = 40, p = 0.752; Fig. 6.1, Exp. 2). Similarly, oven bags enclosing a frame with the excised wrapped-up section of a web or a frame with the corresponding intact remainder of that same web were entered first equally often by males ($\chi^2 = 2.95$, df = 1, n = 41, p = 0.086; Fig. 6.1, Exp 3). No experimental side bias of males was observed ($\chi^2 = 0$, df = 1, n = 20, p = 0.500; Fig. 6.1).

Males engaged in web reduction behavior only on frames bearing an intact web but not on empty control frames ($\chi^2 = 32.72$, df = 1, n = 41, $p < 0.001$; Fig. 6.2, Exp. 1). A similar proportion of males web-reduced on frames bearing an intact web and on frames bearing a reduced web ($\chi^2 = 0.89$, df = 1, n = 40, p = 0.345; Fig. 6.2, Exp. 2). However, when offered a choice between frames bearing the excised wrapped-up section of a web or the remainder intact section of that same web, males engaged in web-reduction behavior only on the intact remainder of the web ($\chi^2 = 20.43$, df = 1, n = 41, $p < 0.001$; Fig. 6.2, Exp. 3).

6.4.2 H2: Deposition of silk by courting males has an inter-sexual signal function that enhances their likelihood of copulation (Exp. 5)

Fourteen out of 40 female-male pairs copulated. Ten of these 14 pairs involved males with functional spinnerets, making them more likely to copulate than males with dysfunctional spinnerets (Table 6.1). The functionality of the males' spinnerets had an effect on (i) the time males spent web-reducing (Table 6.1), but not on the latency to copulation (Table 6.1) and the time spent in copula (Table 6.1). Most web reduction behavior and most copulations occurred on the sheet area [55] of the webs.

The likelihood of males with functional or dysfunction spinnerets to copulate with the female they courted increased with increasing time they spent web-reducing ($\chi^2 = 10.97$, df = 1, $p < 0.001$; Fig. 6.3, Exp. 5). However, the time males spent web-reducing had no effect on the latency to copulation ($F_{1,13} = 0.67$, p = 0.430; Fig. 6.3, Exp. 5).

6.4.3 H3: Stridulatory sound is a courtship signal of males (Exp. 6–7)

The stridulatory apparatus of *S. grossa* males in a North American population closely resembled that of males in a European population [38,40]. SEM images of males from North America revealed tooth-like structures on the abdomen (Fig. 6.4a) that could be scraped over ridges on the prosoma (Fig. 6.4b), thus producing sound.

The mean (± SE) time for the up- and down-movement of the male's abdomen was 0.195 ± 0.011 s and 0.219 ± 0.026 s, respectively (Exp. 7). Sound recordings during abdominal movements of males when stridulatory sound may occur revealed no sound in the frequency range of 0-11 kHz that could have served as courtship signals. Background noise sound recordings indicated frequency components in the range of 0.5-1.5 kHz, 3-4 kHz and 7 kHz at very low levels and entirely dissimilar to the stridulatory sound produced by European *S. grossa* males. Thirteen out of 20 female-male pairs recorded in the context of H3 copulated, and no male was cannibalized.

6.5 Discussion

We show data indicating that web reduction behavior by *Steatoda grossa* males in North America has no long-range (sensu [56]) affect on mate-seeking males. Instead, web reduction behavior has an inter-sexual (male-female) signaling function that appears to be linked to functional spinnerets of the courting male. The inter-sexual signaling function seems to be based on a silk-borne pheromonal signal produced by the courting male but may also modulated by the amount of time males engage in web reduction causing web vibrations. Males did not produce any stridulatory sound during courtship or copulation, although they possess the stridulatory apparatus for sound production. Below, we elaborate on these conclusions, using the three hypotheses as subheadings.

6.5.1 H1: Web reduction by males renders webs less attractive to rival males.

Webs of *S. grossa* females reduced by a courting male were as attractive to other males as intact webs, indicating that web-reduction behavior has no long-range effect on mate-seeking males. Moreover, the intact section of reduced webs continued to prompt web-reduction by late-arriving males. These results are surprising considering the first sperm precedence in the entelegyne *S. grossa* [34,57]. To recognize a reduced web from

a distance would be adaptive for mate-seeking males as they would save energy avoiding webs of a female that has already mated. In *L. hesperus*, reduced webs are indeed significantly less attractive to males than intact webs [13]. As *L. hesperus* and *S. grossa* males exhibit the same type of web-reduction behavior and achieve comparable results in the form of reduced webs, it is perplexing that reduced *S. grossa* webs remain attractive to males. It seems that web-reducing behavior of *S. grossa* males may have only recently evolved [32,33,38], and that this behavior has not yet curtailed long-range attraction of mates, as it has in *L. hesperus* [13]. Evidence for an evolving communication system in *S. grossa* stems from five studies with spiders originating from the same geographic location in Europe. Early (past-century) studies [36,38,39] report stridulatory courtship of males without web-reducing behavior, whereas more recent (2004, 2018) studies [33,34] report both stridulatory sound and web reduction.

Our observations that *S. grossa* males did not reduce already reduced sections of a web imply that they sensed male pheromone on male silk upon contact, male silk impeded access to female silk bearing female contact sex pheromone, or both.

6.5.2 H2: Deposition of silk by courting males has an inter-sexual signal function that enhances their likelihood of copulation.

The differential success of males with functional and dysfunctional spinnerets in securing a copulation with the female they courted suggests that male silk and silk-borne pheromone, respectively, serves as a male-female sexual communication signal. Web reducing has previously been recognized as an essential element of courtship behavior of (false) black widow males that affects their likelihood of copulating with the courted female [30,32, this study]. However, the relative contributions of the vibratory signals associated with the cutting of the female's web and the male's silk used to bundle the cut-up sections, remained unknown. As males with functional and dysfunctional spinnerets exhibited visually comparable web-reduction behavior, but mostly the males that could disseminate silk secured copulations, it follows that male silk enhances a female's receptivity. However, because males with functional spinnerets spent more time reducing and thus vibrating webs than males with dysfunctional spinnerets, it is still conceivable

that both male silk and the extend of web vibrations affect the female's receptivity and the male's likelihood of copulation with the courted female. As not every female that eventually copulated made physical contact with the male's silk, it follows that it is likely a silk-borne volatile male pheromone that – alone or in combination with vibratory signals – renders the female receptive. Given the rather small amount of silk that the male deposits on a female's web during courtship, it is not surprising that only a single male pheromone (*Z*-9-tricosene) has been identified to date [58].

Curiously, the ability to, or not to, deposit silk during courtship had no effect on other aspects of courtship behavior and interactions between the female and the male, such as cannibalism of males by females, the latency to copulation and the copulation duration. Males with the superglue control-treatment (Exp. 5), and those without superglue application (Exp. 6), secured similar numbers of copulations (10 of 20 and 13 of 20 pairs, respectively), indicating that superglue had no adverse effects on the courtship success of males.

6.5.3 H3: Stridulatory sound is a courtship signal of males.

Male stridulatory signals are part of the courtship repertoire in European populations of *S. grossa* [33,38] but were absent in a North American population of *S. grossa* (this study). Sound recordings revealed no evidence that courting or copulating males produced sound remotely resembling that previously reported for *S. grossa* males in a European population [38]. Abdominal movements of males that seemed suggestive of causing stridulatory sound [32] were too slow (by 2 orders of magnitude), and the angle of movements seemed too shallow [see 38], to produce stridulatory courtship sound resembling that of *S. grossa* males in a European population [38]. Males "being silent" during courtship in our study may explain the relatively low number of copulations (27 out of 60 pairs) that were observed. If females still anticipated stridulatory sound from courting males, then all these silent males would have been appraised inferior prospective mates. It now would be intriguing to study the incidence of copulation in an experiment with a full factorial design, each replicate involving four male-female pairs: two pairs with both the female and the male originating from the same population in Europe or in

North America, and two pairs with the female or the male selected from the European or the North American population.

The reason why *S. grossa* males from the North American population we studied here are silent, not using their stridulatory apparatus during courtship, is unclear. Typically, courtship sound is selected against when it draws the attention of potential predators, as shown in crickets [59], but there is no prevalent predator of *S. grossa*, or other spiders, in North America known to exploit spider courtship sound as a prey location cue. There is also no apparent selection pressure for the evolution of sound as an honest male courtship signal because sound production requires minimal nutritional energy and thus, unlike silk production and deposition by males, is not indicative of a male's physical condition [23,24]. However, there may be selection pressure for male *S. grossa* to shift from sound to chemical communication during courtship in the noisy urban soundscape that is typically inhabited by *S. grossa*. That courting *S. grossa* males exclusively deposited silk (chemical communication) or stridulated (sound communication) [33] supports the concept that this type of shift in communication modality may well be under way. Alternatively, a "genetic bottleneck" in the invaded North American range could have prompted a shift in courtship signaling, especially if stridulation is encoded by a few major loci rather than many minor loci [60]. Such a bottleneck can be expected in the noisy urban soundscape inhabited by *S. grossa* [19–22].

6.6. Conclusion

In the invaded North American range, web reduction behavior by *S. grossa* males has no long-range effect on mate-seeking males. For these males, it would be adaptive to avoid reduced webs occupied by mated females. Yet, reduced webs remained as attractive as intact webs occupied by virgin females, implying that web-reduction behavior by males has only recently evolved and that a "reduced mate competition" function of this behavior is not yet established. However, web reduction behavior by *S. grossa* males does have an inter-sexual (male-female) signaling function that appears to be linked to functional spinnerets of the courting male. The inter-sexual signaling function seems to be based on a silk-borne pheromonal signal produced by the courting

male but may also be shaped by the extent of time during which web vibrations caused by web reduction occur. Males did not produce any stridulatory sound during courtship or copulation, although they possess the stridulatory apparatus for sound production.

6.7 Acknowledgments

We thank four anonymous reviewers, particularly reviewer #3, for meticulous reviews and constructive comments, and Derrick Horne and staff of the UBC BioImaging Facility for assistance with scanning electron microscopy. The research was supported by a Graduate Fellowship from Simon Fraser University and the McCarthy Bursary to AF, by an Undergraduate Student Research Award from the Natural Sciences and Engineering Research Council of Canada (NSERC) to JLSV and by an NSERC – Industrial Research Chair to GG, with Scotts Canada Ltd. as the industrial sponsor. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

6.8 References

1. Mateos C, Carranza J. Effects of male dominance and courtship display on female choice in the ring-necked pheasant. Behavioral Ecology and Sociobiology. 1999; 45:235–244.
2. Umbers KDL, Symonds MRE, Kokko H. The mothematics of female pheromone signaling: Strategies for aging virgins. The American Naturalist. 2015; 185:417–432. doi:10.1086/679614
3. Steiger S, Whitlow S, Peschke K, Muller JK. Surface chemicals inform about sex and breeding status in the biparental burying beetle *Nicrophorus vespilloides*. Ethology. 2009; 115:178–185. doi:10.1111/j.1439-0310.2008.01600.x
4. Steiger S, Gershman SN, Pettinger AM, Eggert A-K, Sakaluk SK. Dominance status and sex influence nutritional state and immunity in burying beetles *Nicrophorus orbicollis*. Behavioral Ecology. 2012; 23:1126–1132. doi:10.1093/beheco/ars082
5. Scott C, Anderson AG, Andrade MCB. A review of the mechanisms and functional roles of male silk use in spider courtship and mating. Journal of Arachnology. 2018; 46:173–206.
6. Wyatt TD. Pheromones and animal behavior: chemical signals and signatures. 2nd ed. Cambridge University Press; 2014.

7. Miles MC, Fuxjager MJ. Synergistic selection regimens drive the evolution of display complexity in birds of paradise. Journal of Animal Ecology. 2018; 87:1149–1159. doi:10.1111/1365-2656.12824
8. Simmons LW, Ritchie MG. Symmetry in the songs of crickets. Proceedings of the Royal Society B: Biological Sciences. 1996; 263:1305–1311. doi:10.1098/rspb.1996.0191
9. Zaspel JM, Weller SJ, Wardwell CT, Zahiri R, Wahlberg N. Phylogeny and evolution of pharmacophagy in tiger moths (Lepidoptera: Erebidae: Arctiinae). PLoS ONE. 2014; 9:e101975. doi:10.1371/journal.pone.0101975
10. Vibert S, Scott C, Gries G. A meal or a male: the “whispers” of black widow males do not trigger a predatory response in females. Frontiers in Zoology. 2014; 11:4. doi:10.1186/1742-9994-11-4
11. Vasconcelos RO, Simões JM, Almada VC, Fonseca PJ, Amorim MCP. Vocal behavior during territorial intrusions in the lusitanian toadfish: Boatwhistles also function as territorial “keep-out” signals. Ethology. 2010; 116:155–165. doi:10.1111/j.1439-0310.2009.01722.x
12. Dabelsteen T, McGregor PK, Holland J, McGregor PK, Lampe HM, Langmore NE. Quiet song in song birds: An overlooked phenomenon. Bioacoustics. 1998; 9:89–105. doi:10.1080/09524622.1998.9753385
13. Scott C, Kirk D, McCann S, Gries G. Web reduction by courting male black widows renders pheromone-emitting females’ webs less attractive to rival males. Animal Behaviour. Elsevier Ltd; 2015; 107:71–78. doi:10.1016/j.anbehav.2015.06.009
14. Balsby TJS, Dabelsteen T. Male calling between courtship sequences in whitethroats: a way to counter intrusions from neighbouring rivals. Behavioural Processes. 2003; 63:149–157. doi:10.1016/S0376-6357(03)00052-4
15. Van Dyk DA, Evans CS. Opponent assessment in lizards: examining the effect of aggressive and submissive signals. Behavioral Ecology. 2008; 19:895–901. doi:10.1093/beheco/arn052
16. Yorzinski JL, Patricelli GL, Bykau S, Platt ML. Selective attention in peacocks during assessment of rival males. Journal of Experimental Biology. 2017; 220:1146–1153. doi:10.1242/jeb.150946
17. Dowling JL, Luther DA, Marra PP. Comparative effects of urban development and anthropogenic noise on bird songs. Behavioral Ecology. 2012; 23:201–209. doi:10.1093/beheco/arr176
18. Luther D, Baptista L. Urban noise and the cultural evolution of bird songs. Proceedings of the Royal Society B: Biological Sciences. 2010; 277:469–473. doi:10.1098/rspb.2009.1571
19. Vandergast AG, Lewallen EA, Deas J, Bohonak AJ, Weissman DB, Fisher RN. Loss of genetic connectivity and diversity in urban microreserves in a southern California endemic Jerusalem cricket (Orthoptera: Stenopelmatidae: *Stenopelmatus* n. sp. “santa monica”). Journal of Insect Conservation. 2009; 13:329–345. doi:10.1007/s10841-008-9176-z
20. Schäfer MA, Hille A, Uhl GB. Geographical patterns of genetic subdivision in the cellar spider *Pholcus phalangioides* (Araneae). Heredity. 2001; 86:94–102. doi:10.1046/j.1365-2540.2001.00815.x

21. Fauvelot C, Bertozzi F, Costantini F, Airoldi L, Abbiati M. Lower genetic diversity in the limpet *Patella caerulea* on urban coastal structures compared to natural rocky habitats. Marine Biology. 2009; 156:2313–2323. doi:10.1007/s00227-009-1259-1
22. Dronnet S, Chapuisat M, Vargo EL, Lohou C, Bagnères AG. Genetic analysis of the breeding system of an invasive subterranean termite, *Reticulitermes santonensis*, in urban and natural habitats. Molecular Ecology. 2005; 14:1311–1320. doi:10.1111/j.1365-294X.2005.02508.x
23. Harari AR, Steinitz H. The evolution of female sex pheromones. Current Zoology. 2013; 59:569–578.
24. Davies NB, Krebs JR, West SA. An introduction to behavioural ecology. 4th ed. West Sussex, UK: Wiley-Blackwell; 2012.
25. Galeotti P, Sacchi R, Fasola M, Rosa DP, Marchesi M, Ballasina D. Courtship displays and mounting calls are honest, condition-dependent signals that influence mounting success in Hermann's tortoises. Canadian Journal of Zoology. 2005; 83:1306–1313. doi:10.1139/z05-130
26. Laidre ME, Johnstone RA. Animal signals. Current Biology. Elsevier; 2013; 23:829–833. doi:10.1016/j.cub.2013.07.070
27. Scott C, Vibert S, Gries G. Evidence that web reduction by western black widow males functions in sexual communication. The Canadian Entomologist. 2012; 144:672–678. doi:10.4039/tce.2012.56
28. DiRienzo N, Bradley CT, Smith CA, Dornhaus A. Bringing down the house: male widow spiders reduce the webs of aggressive females more. Behavioral Ecology and Sociobiology. Behavioral Ecology and Sociobiology; 2019; 73:1–10.
29. Watson PJ. Transmission of a female sex pheromone thwarted by males in the spider *Linyphia litigiosa* (Linyphiidae). Science. 1986; 233:219–221.
30. Fischer A. Chemical communication in spiders - a methodological review. Journal of Arachnology. 2019; 47:1–27.
31. Liu J, May-Collado LJ, Pekár S, Agnarsson I. A revised and dated phylogeny of cobweb spiders (Araneae, Araneoidea, Theridiidae): A predatory cretaceous lineage diversifying in the era of the ants (Hymenoptera, Formicidae). Molecular Phylogenetics and Evolution. 2016; 94:658–675. doi:10.1016/j.ympev.2015.09.023
32. Scott C, Gerak C, McCann S, Gries G. The role of silk in courtship and chemical communication of the false widow spider, *Steatoda grossa* (Araneae: Theridiidae). Journal of Ethology. Springer Japan; 2018; 36:191–197. doi:10.1007/s10164-017-0539-3
33. Knoflach B. Diversity in the copulatory behaviour of comb-footed spiders (Araneae, Theridiidae). Denisia. 2004; 12:161–256.
34. Welzenberger R. Paarungsverhalten und Fortpflanzungssystem der kosmopolitischen Kugelspinne *Steatoda grossa* (Araneae , Theridiidae). Leopold-Franzens-Universität Innsbruck. 2018.
35. Gerhardt U. Neue Sexualbiologische Spinnenstudien. Zoomorphology. 1924;567–618.
36. Gerhardt U. Neue sexualbiologische Spinnenstudien. Zoomorphology. 1925; 3:567–618. doi:10.1017/CBO9781107415324.004

37. Gerhardt U. Weitere Untersuchungen zur Biologie der Spinnen. Zoomorphology. 1926; 6:1–77.
38. Gwinner-Hanke H. Zum Verhalten zweier stridulierender Spinnen *Steatoda bipunctata* Linne und *Teutana grossa* Koch (Theridiidae, Araneae), unter besonderer Berücksichtigung des Fortpflanzungsverhaltens. Ethology. 1970; 27:649–678. doi:10.1111/j.1439-0310.1970.tb01893.x
39. Braun R. Zur Biologie von *Teutana triangulosa*. Zeitschrift für wissenschaftliche Zoologie. 1956; 159:255–318.
40. Lee RCP, Nyffeler M, Krelina E, Pennycook BW. Acoustic communication in two spider species of the genus *Steatoda* (Araneae, Theridiidae). Mitteilungen der Schweizerischen Entomologischen Gesellschaft. 1986; 59:337–348. doi:10.3929/ethz-a-005779308
41. Levi HW. The spider genera *Crustulina* and *Steatoda* in North America, Central America, and the West Indies (Araneae: Theridiidae). Bulletin of the Museum of Comparative Zoology. 1957; 117:367–424.
42. Bradley RA. Common Spiders of North America. Edition 1st, editor. University of California Press; 2012.
43. Fischer A, Lee Y, Stewart J, Gries G. Dodging sexual conflict? – Sub-adult females of a web-building spider stay cryptic to mate-seeking adult males. Ethology. 2018; 124:838–843.
44. Kasumovic MM, Andrade MCB. Male development tracks rapidly shifting sexual versus natural selection pressures. Current Biology. 2006; 16:242–243. doi:10.1016/j.cub.2006.03.017
45. Cory A-L, Schneider JM. Effects of social information on life history and mating tactics of males in the orb-web spider *Argiope bruennichi*. Ecology and Evolution. 2017; 8:344–355. doi:10.1002/ece3.3672
46. Derstine NT, Ohler B, Jimenez SI, Landolt P, Gries G. Evidence for sex pheromones and inbreeding avoidance in select North American yellowjacket species. Entomologia Experimentalis et Applicata. 2017; 164:35–44. doi:10.1111/eea.12591
47. Fischer A, Ayasse M, Andrade MCB. Natural compounds as spider repellents: Fact or myth? Journal of Economic Entomology. 2018; 111:314–318. doi:10.1093/jee/tox339
48. Zhang S, Kuntner M, Li D. Mate binding: Male adaptation to sexual conflict in the golden orb-web spider (Nephilidae: *Nephila pilipes*). Animal Behaviour. 2011; 82:1299–1304. doi:10.1016/j.anbehav.2011.09.010
49. Schulte-Hostedde AI, Zinner B, Millar JS, Hickling GJ. Restitution of mass – size residuals: Validating body condition indices. Ecology. 2005; 86:155–163. doi:doi:10.1890/04-0232
50. Cory A-L, Schneider JM. Old maids have more appeal: effects of age and pheromone source on mate attraction in an orb-web spider. PeerJ. 2016; 4:e1877. doi:10.7717/peerj.1877
51. Mistal C, Takacs S, Gries G. Evidence for sonic communication in the german cockroach. The Canadian Entomologist. 2000; 132:867–876.
52. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2018.

53. Fischer A. Function of web reduction behavior in a North American population of the false black widow spider. In: Dryad data repository. 2019. doi:10.5061/dryad.dbrv15dwx
54. Bates D, Machler M, Bolker BM, Walker SC. Fitting linear mixed-effects models using lme4. Journal of Statistical Software. 2015; 67:1–48.
55. Blackledge TA, Zevenbergen JM. Condition-dependent spider web architecture in the western black widow*, Latrodectus hesperus*. Animal Behaviour. 2007; 73:855–864. doi:10.1016/j.anbehav.2006.10.014
56. Roelofs WL, Carde RT. Responses of Lepidoptera to synthetic sex pheromone chemicals and their analogues. Annual Review of Entomology. 1977; 22:377–405.
57. Segev O, Ziv M, Lubin Y. The male mating system in a desert widow spider. Journal of Arachnology. 2003; 31:379–393. doi:10.1636/s01-101
58. Xiao Y-H, Zhang J-X, Li S-Q. Male-specific (Z)-9-tricosene stimulates female mating behaviour in the spider *Pholcus beijingensis*. Proceedings of the Royal Society B. 2010; 277:3009–3018. doi:10.1098/rspb.2010.0763
59. Zuk M, Rotenberry JT, Tinghitella RM. Silent night: adaptive disappearance of a sexual signal in a parasitized population of field crickets. Biology Letters. 2006; 2:521–524. doi:10.1098/rsbl.2006.0539
60. Arbuthnott D. The genetic architecture of insect courtship behavior and premating isolation. Heredity. Nature Publishing Group; 2009; 103:15–22. doi:10.1038/hdy.2009.22

Table 6.1: Criteria recorded during courtship of 40 male-female *Steatoda grossa* pairs, with 20 males having dysfunctional (experimentally occluded) spinnerets and 20 males having functional (intact) spinnerets. The table shows either the number of occurrences or the mean ± standard error, together with significance testing of the spinneret treatment effect (from generalized mixed models). The calculated means for latency to, and duration of, copulation were calculated only for males that copulated, whereas means of web-reduction behavior include data from all males.

	Spinnerets		
Criteria recorded	Dysfunctional	Functional	Statistical results
Copulations	4	10	$\chi^2 = 4.72$, df = 1, p = 0.03
Web-reducing	17	20	$\chi^2 = 3.24$, df = 1, p = 0.07
Mean ± SE time males spent web-reducing	346 ± 119 s	899 ± 149 s	$\chi^2 = 10.91$, df = 1, p = 0.001
Mean ± SE latency to copulation	3311 ± 1943 s	4989 ± 2299 s	$F_{1,13} = 0.53$, p = 0.48
Mean ± SE duration of copulation	1298 ± 577 s	1000 ± 682 s	$F_{1,13} = 0.19$, p = 0.67
Female cannibalism	1	1	$\chi^2 = 0.00$, df = 1, p = 1

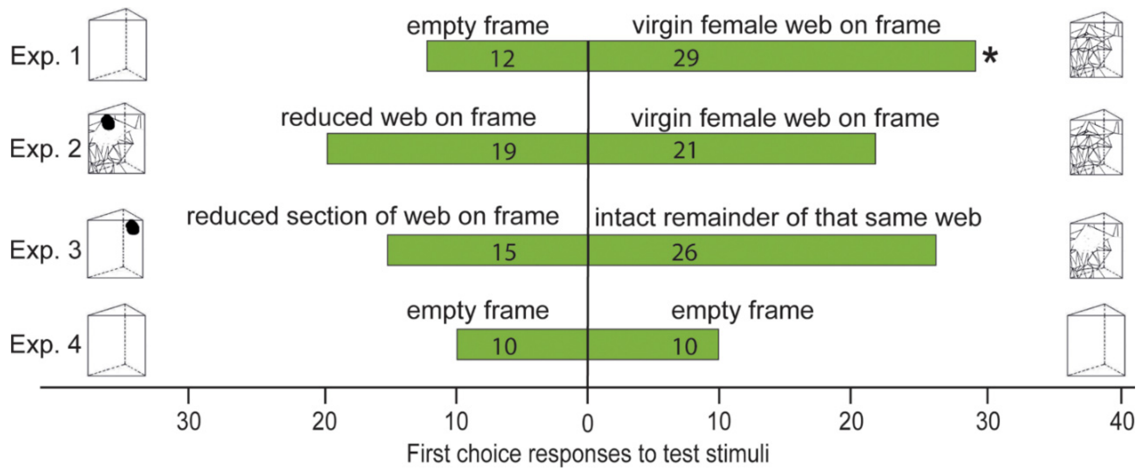


Figure 6.1: Anemotactic attraction of male *Steatoda grossa*. First-choice responses of males to specific test stimuli in Y-tube olfactometer experiments 1 (n = 41), 2 (n = 40), 3 (n = 41) and 4 (n = 20). Numbers in bars indicate the number of males choosing the respective stimulus. One male did not respond in Exp. 2. The asterisk (*) denotes a significant preference for the respective stimulus; χ^2 test; $p < 0.05$.

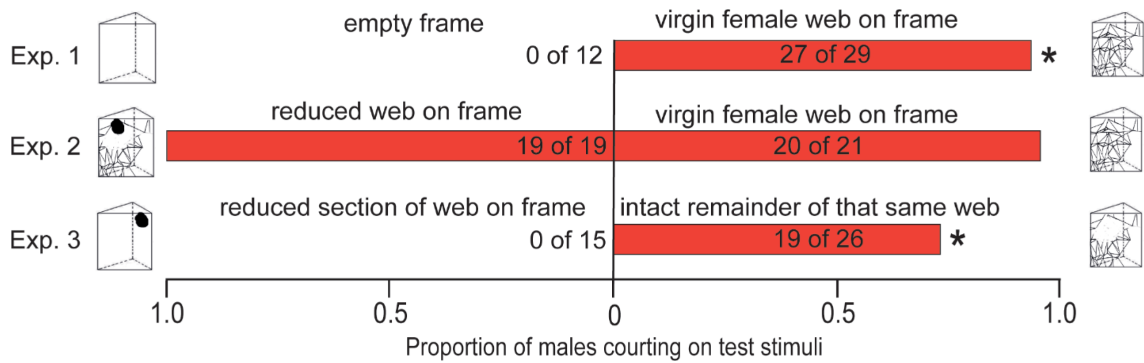


Figure 6.2: **Occurrence of web reduction by male *Steatoda grossa*.** Proportion of *Steatoda grossa* males engaging in web reduction behavior (element of courtship display) in response to test stimuli. In each of experiments 1 (n = 41), 2 (n = 40), and 3 (n = 41), the asterisk (*) denotes a significant preference for the respective stimulus; χ^2 test; $p < 0.05$.

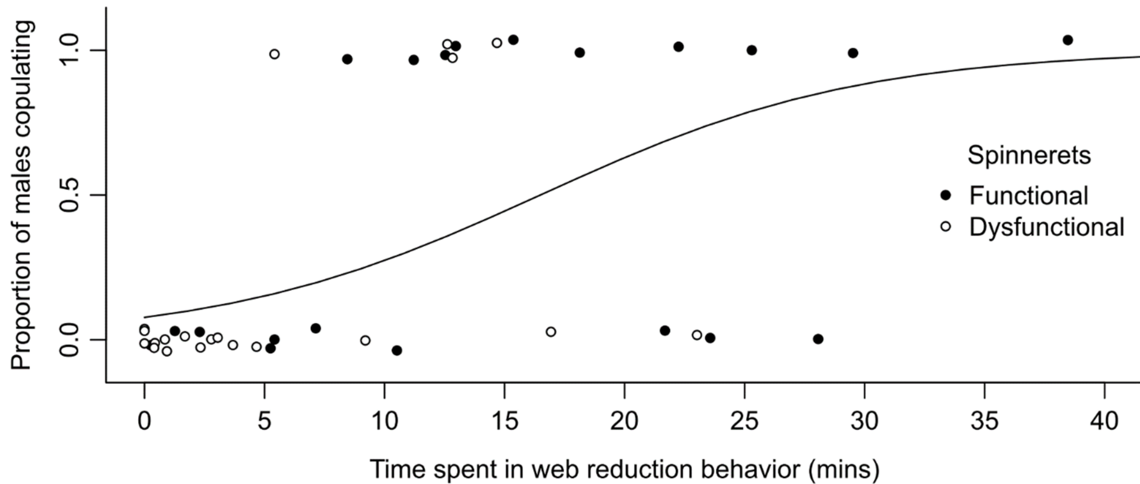


Figure 6.3: Web-reduction by male Steatoda grossa and likelihood of copulation. The likelihood of males with functional or dysfunctional spinnerets to copulate with the female they courted increased with increasing time they engaged in web reduction behavior; general linear mixed model, $p < 0.001$; the line shows the predicted likelihood of copulation in relation to the time spent in web-reduction. Female aggression towards males was not affected by the time males spent web-reducing ($\chi^2 = 0.37$, df = 1, $p = 0.54$). One male with functional spinnerets and one male with dysfunctional spinnerets was cannibalized by the courted female.

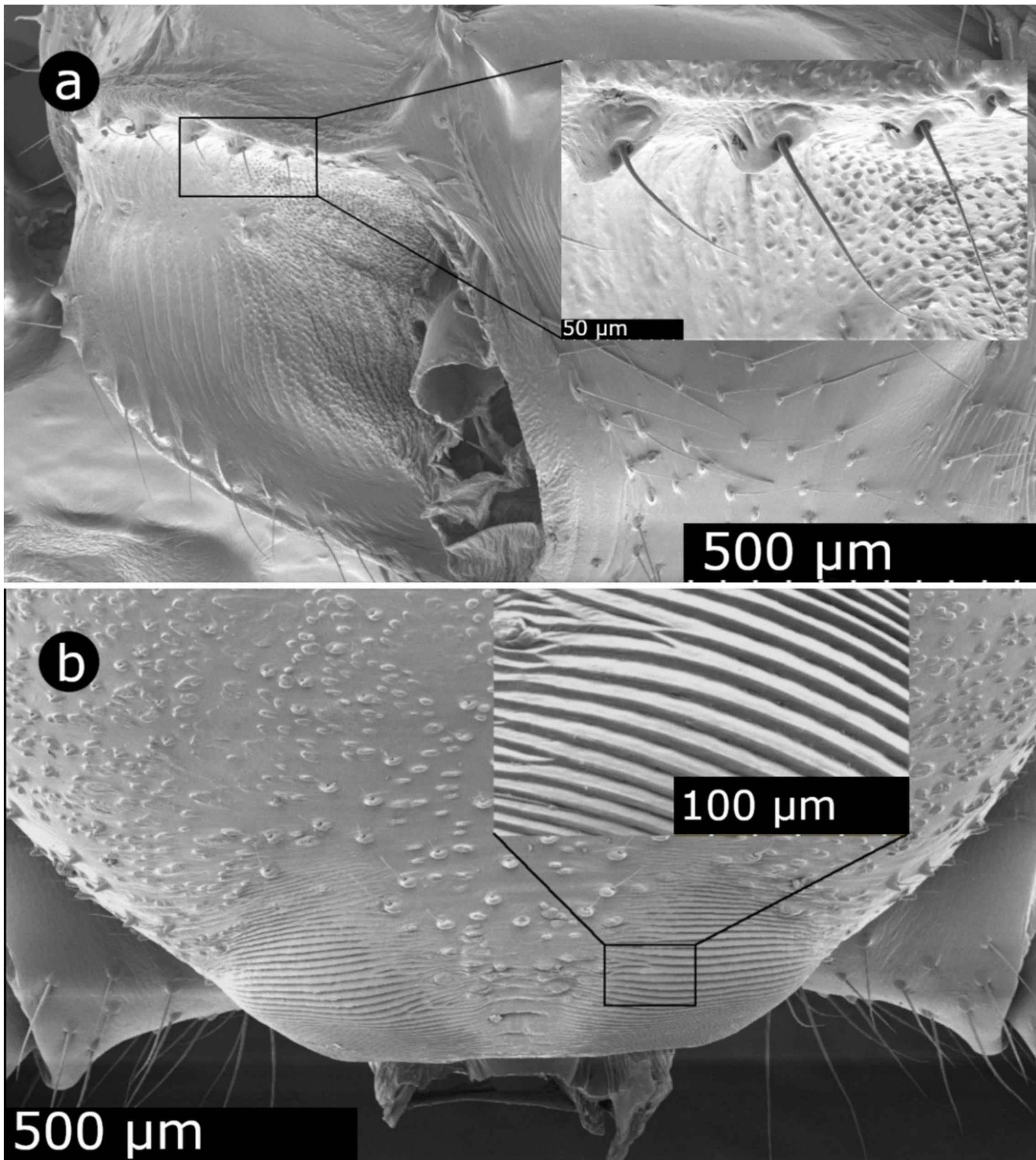


Figure 6.4: **Stridulatory apparatus of a male *Steatoda grossa*.** Scanning electron micrographs show (a) teeth-like structures (the scraper) on the anterior ventrum of the abdomen, and (b) ridges (the file) on the posterior tergum of the prosoma (cephalothorax).

Chapter 7: 'Mine or Thine' – Indiscriminate responses to own and conspecific webs and egg sacs by the false black widow spider, *Steatoda grossa* (Araneae:Theridiidae) [1]

[1]The corresponding manuscript has been published in the *Journal of Ethology* (Volume 38, 241–245, 2020), with the following authors: Andreas Fischer, Emmanuel Hung, Neilofar Amiri, Gerhard Gries

7.1 Abstract

Female false black widows, *Steatoda grossa* (Araneae: Theridiidae), build energy-costly webs. We tested the hypotheses (H1, H2) that females prefer their own webs and the chemical extract of their own webs to those of conspecifics, and (H3) that mated females discern their own egg sacs and that of conspecifics. In choice bioassays, females indiscriminately accepted both their own webs and egg sacs and those of conspecific females, although they chose extracts of webs based on their chemical cues. The females' indiscriminate responses to webs or egg sacs are likely due to a lack of selection pressure to reject webs or egg sacs of conspecific females.

7.2 Introduction

Animal-built structures, such as beaver dams, the Brants' whistling rat burrow system, leafcutter ants' nests and the webs of spiders, provide shelter, aid prey capture or foraging, and facilitate communication (Hansell 2005). Structures differ in functional integrity and efficiency in accordance with the physiological state of the "architect" (Blackledge and Zevenbergen 2007), thus providing incentive for individuals to discern "mine or thine".

The webs of web-building spiders function as shelter, prey capture, courtship and egg-sac guarding sites (Foelix 2015). Web construction by spiders is energy-costly, requiring 4.5 cal for 1 mg of silk (Tanaka 1989). While orb-weavers build their 2-dimensional webs over the period of a few hours and mitigate silk production costs by consuming their old silk (Opell 1998), cobweb-weaving spiders build their semi-permanent, 3-dimensional webs over several days without "recycling" their silk (Janetos 1982). Webs are also a fitness investment for mated theridiid females in that they provide a "safe haven" for their egg sacs (Foelix 2015).

Adult females of most web-building spiders remain sessile on their webs. When threatened, they often drop out of their webs to avoid predation (Uma and Weiss 2012). If their webs are destroyed during attempted predation or through a mechanical disturbance, they may rebuild their web or settle elsewhere on existing webs which are indicative of quality habitats (reviewed in Fischer 2019), as we have recently shown for mated females of the false black widow spider, *Steatoda grossa* C. L. Koch 1838 (Theridiidae) (Fischer et al. 2019). However, selecting a web still occupied by a conspecific female may result in conflict or even cannibalism (Wise 2006). Whether displaced theridiids are able to discern their own webs and conspecific webs is not yet known.

A displaced, previously egg sac-guarding female spider would accrue fitness benefits from recognizing and returning to her own web. Otherwise, her egg sac would remain undefended in the vacated web and be vulnerable to predation or parasitism (Austin 1985). Recognizing both her own web and egg sac would be a fail-safe mechanism, ensuring the reproductive fitness of a displaced previously egg sac-guarding

female. It is surprising then that females of the brown widow spider, *Latrodectus geometricus* C. L. Koch 1841, did not discern their own egg sac and that of conspecific females (Guimarães et al. 2016). Here we studied web and egg sac recognition by female *S. grossa*. Conceivably, both unique web architecture and signature semiochemicals on the web could mediate web identity recognition. We tested the hypotheses (H1-3) that virgin and mated females prefer their own webs (H1), and the extract of their own webs (H2), to those of conspecifics, and (H3) that mated females discern their own egg sacs and those of conspecifics.

7.3 Materials and methods

7.3.1 Experimental spiders

We collected 78 (mated) female *S. grossa* in hallways of the Burnaby campus of Simon Fraser University (49°16’37.35”N, 122°55’4.65”W), and reared the offspring of these females on a diet of vinegar flies and blow flies as previously described (Fischer et al. 2018). We considered isolated adult females ‘virgins’ when they had molted to maturity in the insectary, and ‘mated’ when they produced an egg sac.

7.3.2 General bioassay design

We followed a protocol previously detailed (Fischer et al. 2019). Briefly, we bio-assayed behavioral responses of both virgin and mated spiders (n = 30 for each type in each of experiments 1–12) to test stimuli, using a large and a small t-shaped climbing structure (Fischer et al. 2019) with a frame of bamboo skewers placed at either end of the horizontal beam (large T-rod bioassays), or with a piece of filter paper stapled to either end of the horizontal beam (small T-rod bioassays). The large T-rod (horizontal beam: 72 × 0.4 cm; vertical beam: 8 × 0.4 cm) and the small T-rod (horizontal beam: 25 × 0.4 cm; vertical beam: 30 × 0.4 cm) were made of bamboo skewers (GoodCook, CA, USA) fixed together with labeling tape (5 × 1.9 cm; Fisher Scientific, Ottawa, ON). The bamboo skewer frames had a prism-like geometry with a triangular equilateral (30 cm) top and bottom and rectangular sides (22 cm high). For each bioassay replicate, we introduced the spider onto the vertical beam of the T-rod under red light and recorded for 15 min the

time she spent on each of the two frames, or on each of the two pieces of filter paper. For each replicate, we alternated the sides test stimuli occupied and replaced any components previously contacted by a spider (Fischer et al. 2019).

7.3.3 Specific experiments

H1: Recognition of web identity (Exps. 1–6)

To obtain webs for bioassays, we placed a female spider on a bamboo frame and allowed her two days to build a web. In experiments 1–6, we offered each bioassay spider a choice between frames bearing (*i*) her own web or the web of a conspecific female (Exps. 1, 2), (*ii*) her own web or no web (Exps. 3, 4), and (*iii*) the web of a conspecific female or no web (Exps. 5, 6).

H2: Recognition of web extract identity (Exps. 7–12)

We obtained and tested web extracts as previously detailed (Fischer et al. 2018). Briefly, we obtained webs of virgin and mated females (see above) and then extracted them individually for 24 h in 50 µl of methanol (99.9 % HPLC grade; Fisher Chemical, ON, Canada). We applied each extract and a corresponding volume of methanol (control stimulus), respectively, to one of the two pieces of filter paper attached to the horizontal arm of the small T-rod (see above). In experiments 7-12, we offered each spider a choice between filter paper treated with (*i*) the extract of her own web or the extract of a conspecific female web (Exps. 7, 8), (*ii*) the extract of her own web or methanol (Exps. 9, 10), and (*iii*) the extract of a conspecific female web or methanol (Exps. 11, 12).

H3: Recognition of egg sac identity (Exps. 13–16)

To determine whether female spiders discern their own egg sac and the egg sac of a conspecific female, we adapted the protocol of Guimarães et al. (2016) for testing egg sac acceptance. We removed egg sacs of mated females from their webs and 24 h later presented these females with either their own egg sac (Exp. 13, n = 30) or the egg sac of a conspecific female (Exp. 14, n = 30). To determine the response of virgin females, we presented them with an egg sac of a conspecific mated female (Exp. 15, n = 30). In each

replicate of experiments 13-15, we presented the egg sac in an inverted petri dish lid (9 × 1 cm), placed a cup (300 ml, Western Family, Canada) housing a female spider over the egg sac, and checked 48 h later whether the female had accepted the egg sac by attaching it to her web.

7.3.4 Data analyses

We worked with the software platform IBM SPSS 23 (UNICOM Systems, CA, USA) for statistical analyses. As data in Exps. 1–12 were not normally distributed; we used a Mann-Whitney-U-Test to analyze the proportion of time spiders spent on test stimuli relative to the total bioassay time. We analyzed egg sac acceptance data (Exps. 13–15) with binomial exact tests.

7.4 Results

7.4.1 H1: Recognition of web identity (Exps. 1–12)

Both virgin and mated females spent similar proportions of time on their own web and the web of a conspecific female (virgin females: n = 30, U = 521.5, P = 0.275; mated females: n = 30, U = 490.5, P = 0.527; Fig. 7.1, Exps. 1, 2). Virgin females spent similar proportions of time on frames bearing their own web and on empty frames (n = 30, U = 425.5, P = 0.705; Fig. 7.1, Exp. 3). Mated females, in contrast, spent more time on frames bearing their own web than on empty frames (n = 30, U = 291, P = 0.015; Fig. 7.1, Exp. 4). Both virgin and mated females spent more time on frames bearing a conspecific web than on empty frames (virgin females: n = 30, U = 267.5, P = 0.005; mated females: n = 30, U = 278.5, P = 0.010; Fig. 7.1, Exps. 5, 6).

7.4.2 H2: Recognition of web extract identity (Exps. 7–12)

Virgin females, but not mated females, spent more time on filter paper treated with web extract of a conspecific female than on filter paper treated with their own web extract (virgin females: n = 30, U = 656.5, P = 0.002; mated females: n = 30, U = 496, P = 0.496; Fig. 7.1, Exps. 7, 8). Both virgin and mated females spent more time on filter paper treated with the extract of their own web than on filter paper treated with methanol

(virgin females: n = 30, U = 258.5, P = 0.005; mated females: n = 30, U = 213, P < 0.001; Fig. 7.1, Exps. 9, 10). However, when offered a choice between filter paper treated with extract of a conspecific web and filter paper treated with methanol, both virgin and mated females spent a similar amount of time on either filter paper (virgin females: n = 30, U = 379, P = 0.294; mated females: n = 30, U = 344.5, P = 0.119; Fig. 7.1, Exps. 11, 12).

7.4.3 H3: Recognition of egg sac identity (Exps. 13–16)

Twenty-six out of 30 mated females accepted their own egg sacs (binomial test, n = 30, P < 0.001; Fig. 7.2, Exp. 13), and 27 out of 30 mated females accepted the egg sacs of conspecific females (binomial test, n = 30, P < 0.001; Fig. 7.2, Exp. 14). Virgin females did not accept any egg sac (binomial test, n = 30, P < 0.001, Fig. 7.2, Exp. 15).

7.5 Discussion

Our data support the conclusions that female *S. grossa* indiscriminately accept both their own web and egg sac and those of conspecific females.

When virgin or mated *S. grossa* females were offered a choice between two frames bearing either their own web or that of a conspecific female, they spent the same proportion of time on either web (Fig. 7.1, Exps. 1, 2), indicating acceptance of either web type. These indiscriminate responses cannot be explained by a lack of web identity cues. Virgin females, when offered a choice between filter paper treated with either an extract of their own web or that of a conspecific female, preferred the latter (Fig. 7.1, Exp. 7), revealing their ability to discern different types of chemical web cues. That this ability was not apparent in choice bioassays with intact webs (Fig. 7.1, Exps. 1, 2) could be attributed to the relative importance of physical and chemical web cues. When the effects of (1) semiochemical-deprived silk micro- and macro-structure (wrapped-up silk or intact web), (2) silk-like material (Halloween spider web decoration) in cobweb arrangement, and (3) silk semiochemical extracts were tested for behavioral responses of *S. grossa* females, it was the web architecture, rather than the web silk or the web semiochemicals, that readily prompted web acceptance and settling responses (Fischer et

al. 2019). Females accepting not only their own web but also that of a conspecific female demonstrated the importance of any pre-existing web as a structural foundation (Fischer et al. 2019) and as an opportunity to mitigate web-building costs.

As silk used for web building and egg sac spinning originate from different silk glands (Kovoor 1987), it was conceivable that egg sac silk carries unique cues that reveal the identity of the maternal female. However, like female *L. geometricus* (Guimarães et al. 2016), female *S. grossa* did not discern their own egg sac and those of conspecific females (Fig. 7.2). These results are surprising in that female spiders can be expected to recognize and defend their own offspring against brood parasites and predators. Spider eggs and spider juveniles are common prey for insects and other spiders (Wise 2006; Vetter et al. 2012). Guarding of egg sacs and spiderlings by female spiders helps alleviate the adverse effects of predation, as shown in various spider taxa (Pollard 1983; Fink 1987; Horel and Gundermann 1992). The reason(s) why *L. geometricus* and *S. grossa* failed to recognize, and ultimately defend, their own egg sacs are not immediately obvious. Lack of selection pressure is one possible explanation. Brood parasitism, where the parasites rely on the host for care of their own offspring, is very rare in spiders (Fischer 2019) and does not seem to exist in *S. grossa*. Therefore, there is no selection pressure for a female *S. grossa* to recognize her own egg sac and to reject that of a conspecific. Guarding exclusively against predators, and rarely encountering an egg sac other than their own, there would again be no selection pressure for female *S. grossa* to recognize and reject egg sacs of conspecific females.

In conclusion, *S. grossa* females do not discern “mine or thine” with respect to webs or egg sacs. Although they seem to sense semiochemical differences between web extracts, their indiscriminate responses to intact webs or egg sacs are likely due to a lack of selection pressure to reject webs or egg sacs of conspecifics.

7.6 Acknowledgments

We thank two anonymous reviewers and the associate editor for constructive comments. The research was supported by a Graduate Fellowship from Simon Fraser

University to A.F., a Vice President Research - Undergraduate Student Research Award to E.H., and by a Natural Sciences and Engineering Research Council of Canada - Industrial Research Chair to G.G., with Scotts Canada Ltd. as the industrial sponsor.

7.7 References

Austin AD (1985) The function of spider egg sacs in relation to parasitoids and predators, with special reference to the australian fauna. Journal of Natural History 19:359–376. https://doi.org/10.1080/00222938500770261

Blackledge TA, Zevenbergen JM (2007) Condition-dependent spider web architecture in the western black widow, *Latrodectus hesperus*. Animal Behaviour 73:855–864. https://doi.org/10.1016/j.anbehav.2006.10.014

Fink LS (1987) Green lynx spider egg sacs: Sources of mortality and the function of female guarding. Journal of Arachnology 15:231–239

Fischer A (2019) Chemical communication in spiders - a methodological review. Journal of Arachnology 47:1–27

Fischer A, Hung E, Gries G (2019) Female false black widow spiders, Steatoda grossa, recognize webs based on physical and chemical cues. Entomologia Experimentalis et Applicata 167:803–810. https://doi.org/10.1111/eea.12825

Fischer A, Lee Y, Stewart J, Gries G (2018) Dodging sexual conflict? – Sub-adult females of a web-building spider stay cryptic to mate-seeking adult males. Ethology 124:838–843. https://doi.org/10.1111/eth.12807

Foelix R (2015) Biologie der Spinnen. Chimaira, Frankfurt am Main

Guimarães IDC, Cardoso CAL, Lima SM, et al (2016) Chemical signals might mediate interactions between females and juveniles of *Latrodectus geometricus* (Araneae: Theridiidae). Behavioural Processes 126:27–35. https://doi.org/10.1016/j.beproc.2016.03.001

Hansell MH (2005) Animal architecture. Oxford University Press, New York

Horel A, Gundermann JL (1992) Egg sac guarding by the funnel-web spider *Coelotes terrestris*: function and development. Behavioural Processes 27:85–94

Janetos AC (1982) Foraging tactics of two guilds of web-spinning spiders. Behavioral Ecology and Sociobiology 10:19–27

Kovoor J (1987) Comparative Structure and Histochemistry of silk-producing organs in Arachnids. In: Ecophysiology of Spiders. Springer, Heidelberg, pp 160–186

Opell BD (1998) Economics of spider orb-webs: the benefits of producing adhesive capture thread and of recycling silk. Functional Ecology 12:613–624

Pollard SD (1983) Egg guarding by *Clubiona cambridgei* (Araneae, Clubionidae) against conspecific predators. Journal of Arachnology 11:323–326. https://doi.org/10.2307/3705040

Tanaka K (1989) Energetic cost of web construction and its effect on web relocation in the web-building spider *Agelena limbata*. Oecologia 81:459–464. https://doi.org/10.1007/BF00378952

Uma DB, Weiss MR (2012) Flee or fight: ontogenetic changes in the behavior of cobweb spiders in encounters with spider-hunting wasps. Environmental Entomology 41:1474–1480. https://doi.org/10.1603/EN12126

Vetter RS, Vincent LS, Itnyre A a., et al (2012) Predators and parasitoids of egg sacs of the widow spiders, *Latrodectus geometricus* and *Latrodectus hesperus* (Araneae: Theridiidae) in southern California. Journal of Arachnology 40:209–214. https://doi.org/10.1636/Hi11-93.1

Wise DH (2006) Cannibalism, food limitation, intraspecific competition, and the regulation of spider populations. Annual Review of Entomology 51:441–465. https://doi.org/10.1146/annurev.ento.51.110104.150947

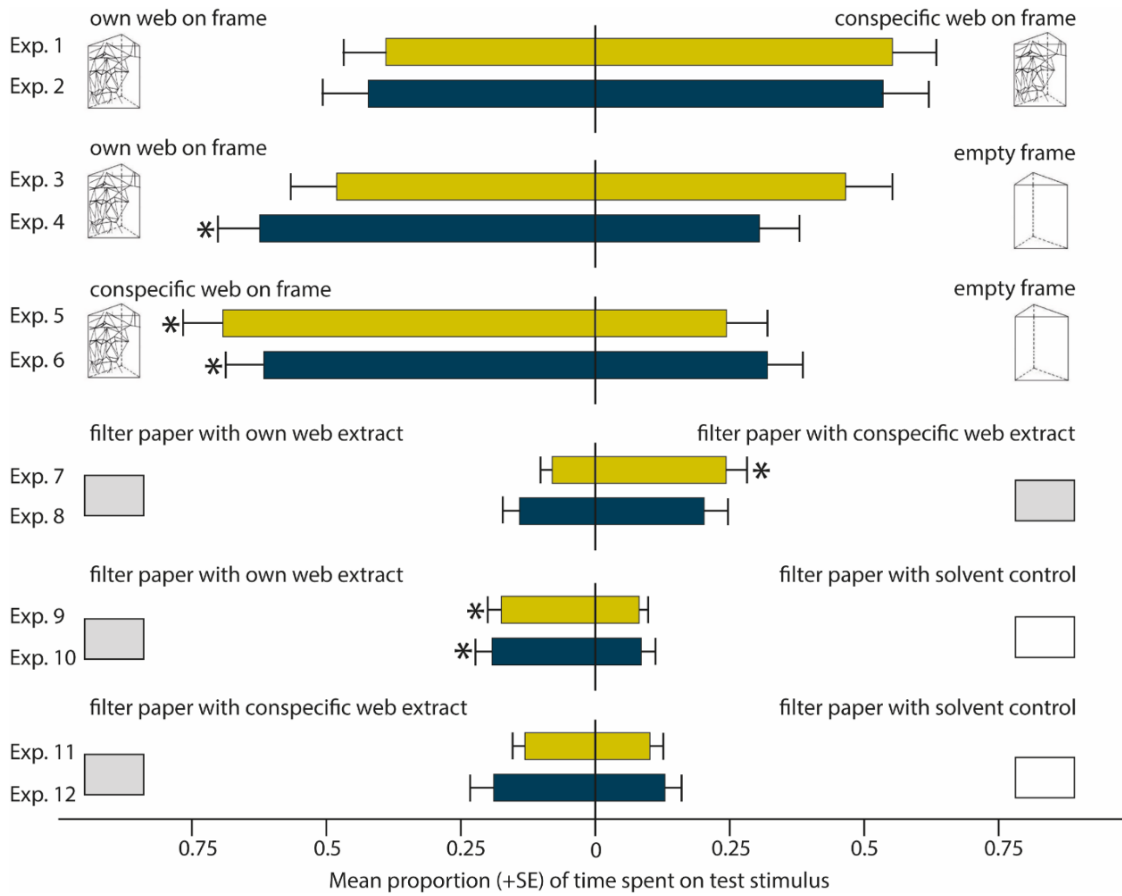


Figure 7.1: **Web acceptance tests.** Mean (+ SE) proportion of time spent by female *Steatoda grossa* on test stimuli. Yellow and blue bars denote virgin and mated females, respectively. In experiments 1-12 (n = 30 for each type of female in each experiment), an asterisk (*) denotes a statistically significant behavioral response to a test stimulus (Mann-Whitney U test, $P < 0.05$).

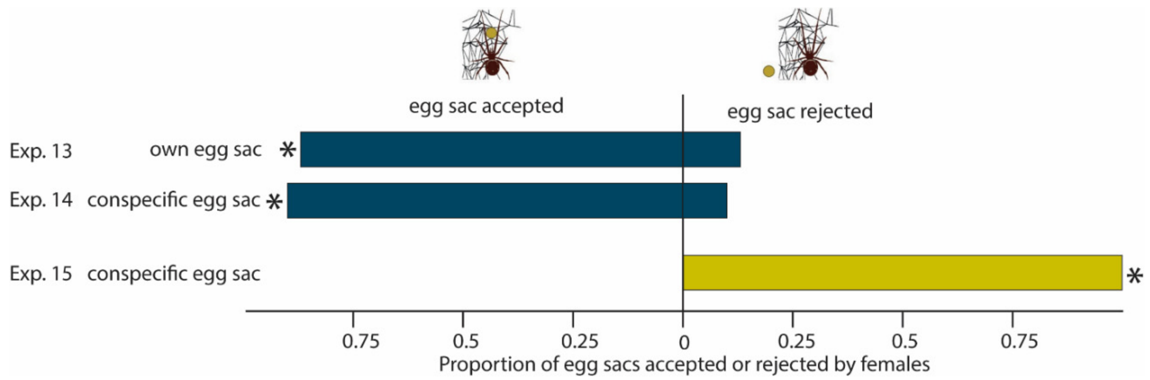


Figure 7.2: **Egg sac acceptance tests.** Proportions of mated females (Exps. 13, 14, n = 30 each) and virgin females (Exp. 15, n = 30) of *Steatoda grossa* accepting or rejecting their own egg sac or the egg sac of a conspecific female. Blue and yellow bars denote mated and virgin females, respectively. An asterisk (*) indicates a statistically significant behavioral response to a test stimulus (binomial test, $P < 0.05$).

Appendix. Supplementary Information of Chapter 3

Table A.1: List of chemicals referred to in this study, with amounts (where applicable) quantified on the web of a female *Steatoda grossa.*

#	Chemical name	Amount per web	Supplier	Purity
1	[(*R*)-3-Hydroxybutyryloxy]-butyric acid	N/A	N/A	N/A
2	*N*-3-Methyl-butyryl-*O*-(*S*)-2-methylbutyryl-L-serine methyl ester	N/A	N/A	N/A
3	*N*-3-Methylbutanoyl-*O*-methylpropanoyl-L-serine methyl ester	N/A	N/A	N/A
4	(*R*)-3-Hydroxybutyric acid	N/A	N/A	N/A
5	Pyrrolidin-2-one	4,000 ng	Sig-Ald[1]	99%
6	4-Hydroxyhydrofuran-2(3*H*)-one	200 ng	Sig-Ald	95%
7	Nonanoic acid	20 ng	Sig-Ald	≥ 97%
8	Dodecanoic acid	100 ng	Sig-Ald	98%
9	6-Methylheptanamide	20 ng	Gries-lab	>95%
10	Octanamide	40 ng	Gries-lab	>95%
11	4,6-dimethylheptanamide	40 ng	Gries-lab	>95%
12	*N*-4-Methylvaleroyl-*O*-butyroyl-L-serine	145 ng	Gries-lab	90%
13	*N*-4-Methylvaleroyl-*O*-butyroyl-L-serine methyl ester[2]	N/A	Gries-lab	N/A
14	*N*-4-Methylvaleroyl-*O*-isobutyroyl-L-serine methyl ester[2]	N/A	Gries-lab	N/A
15	*N*-4-Methylvaleroyl-*O*-hexanoyl-L-serine methyl ester[2]	N/A	Gries-lab	N/A
16	*N*-4-Methylvaleroyl-*O*-isobutyroyl-L-serine	20 ng	Gries-lab	>90%
17	*N*-4-Methylvaleroyl-*O*-hexanoyl-L-serine	20 ng	Gries-lab	>90%
18	*N*-4-Methylvaleroyl-L-serine	200 ng	Gries-lab	>82

19	Butyric acid	103 ng	Sig-Ald	> 99%
20	Isobutyric acid	3 ng	Sig-Ald	99%
21	Hexanoic acid	54 ng	Sig-Ald	> 99%

[1]Sigma-Aldrich
[2]Prepared by diazomethane treatment of **12**, **16** and **17** (compounds were not tested in bioassays)

Table A.2: **Summary of behavioral experiments and analytical procedures.** List of compounds (bold-face) tested and materials analyzed, type of bioassay apparatus [T-rod (Fig. 3.1 c); Y-tube olfactometer (Fig. 3.4 d)] and analytical instruments used for behavioral experiments and chemical analyses, respectively, and statistical procedures applied for data analyses.

Exp. #	Assay/analysis	Compounds[1,2]/material tested	Statistical analyses
Identification of contact pheromone components			
1	T-rod	**5-11**	Wilcoxon rank sum test N = 20, W = 370, $P < 0.001$
2	T-rod	Web extract	
3	T-rod	Web extract	Kruskal-Wallis χ^2 test[4] $\chi^2 = 35.068$, df = 3, $P < 0.001$
4	T-rod	**12**, **16**, **17**	
5	T-rod	**5-11** + **12**, **16**, **17**	
6	T-rod	**5-11**	
7	T-rod	**12**, **16**, **17** (10 FWE)	Kruskal-Wallis χ^2 test[4] $\chi^2 = 61.750$, df = 4, $P < 0.001$
8	T-rod	**12**, **16**, **17** (1 FWE)	
9	T-rod	**12**, **16**, **17** (0.1 FWE)	
10	T-rod	**12**, **16**, **17** (0.01 FWE)	
11	T-rod	**12**, **16**, **17** (0.001 FWE)	
12	T-rod	**12**, **16**, **17**	Kruskal-Wallis χ^2 test[4] $\chi^2 = 11.191$, df = 3, $P = 0.010$
13	T-rod	**12**, **17**	
14	T-rod	**12**, **16**	
15	T-rod	**16**, **17**	
16	T-rod	**12**, **16**	Kruskal-Wallis χ^2 test[4] $\chi^2 = 3.652$, df = 2, $P = 0.160$
17	T-rod	**12**	
18	T-rod	**16**	
Origin of contact pheromone components			
19	HPLC-MS[3]	Spider tagmata	Wilcoxon rank sum test
			N = 22, W = 21, $P = 0.004$
20	HPLC-MS	Abdominal tissues	Kruskal-Wallis χ^2 test[4]
			$\chi^2 = 70.96$, df = 6, $P < 0.001$
21	HPLC-MS	Silk glands	Kruskal-Wallis χ^2 test[4]

			$\chi^2 = 36.00$, df = 6, $P < 0.001$
Transition of contact pheromone components to mate attractant pheromone components			
22	Y-tube	Web extract	One-sided binomial test: $P = 0.013$
23	Y-tube	**5-11**	One-sided binomial test: $P = 0.588$
24	HPLC-MS	**18** / (**18**+**12**+**16**+**17**)	Wilcoxon rank sum test W= 638, N = 70, $P < 0.001$
25	Y-tube	**18-21**	One-sided binomial test: $P = 0.030$
26	Y-tube	**19-21**	One-sided binomial test: $P = 0.006$
27	Y-tube	**18**	One-sided binomial test: $P = 0.500$
28	Adhesive traps in hallways	**19-21**	One-sided binomial test: $P = 0.011$
Mechanisms underlying the transition of contact pheromone components to sex attractant pheromone components			
29	T-rod	**12**, **16**, **17**, **18**	Kruskal-Wallis χ^2 test[4] $\chi^2 = 12.78$, df = 2, $P < 0.001$
30	T-rod	**12**, **16**, **17**	
31	T-rod	**18**	
32	pH / HPLC-MS	webs/web extracts	Generalized linear model $F_{1,69} = 108.44$, $P < 0.001$
33	HPLC-MS	**12** (in pH 7 buffer solution)	Kruskal-Wallis χ^2 test[4] $\chi^2 = 25.84$, df = 2, $P < 0.001$
34	HPLC-MS	**12** (in pH 4 buffer solution)	
35	HPLC-MS	**12** (in acetonitrile)	

[1]Numbers refer to chemicals listed in Appendix Table 3.1
[2]Female web equivalent: amount of analyte present in the extract of a web from a single female *S. grossa*
[3]HPLC-MS: High performance liquid chromatography – mass spectrometry
[4]p-value corrected for multiple comparison using the Bonferroni-Hochberg method.

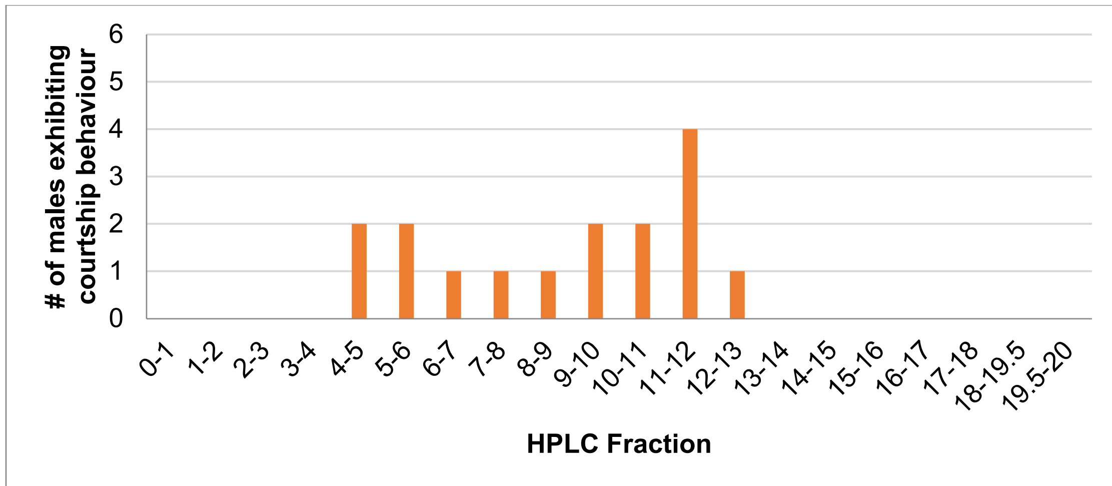


Figure A.1: **Courtship by *Steatoda grossa* males in response to HPLC fractions of female *S. grossa* web extract.** Number of males exhibiting courtship behaviour in response to high performance liquid chromatography (HPLC) fractions of crude extract of female *S. grossa* webs.

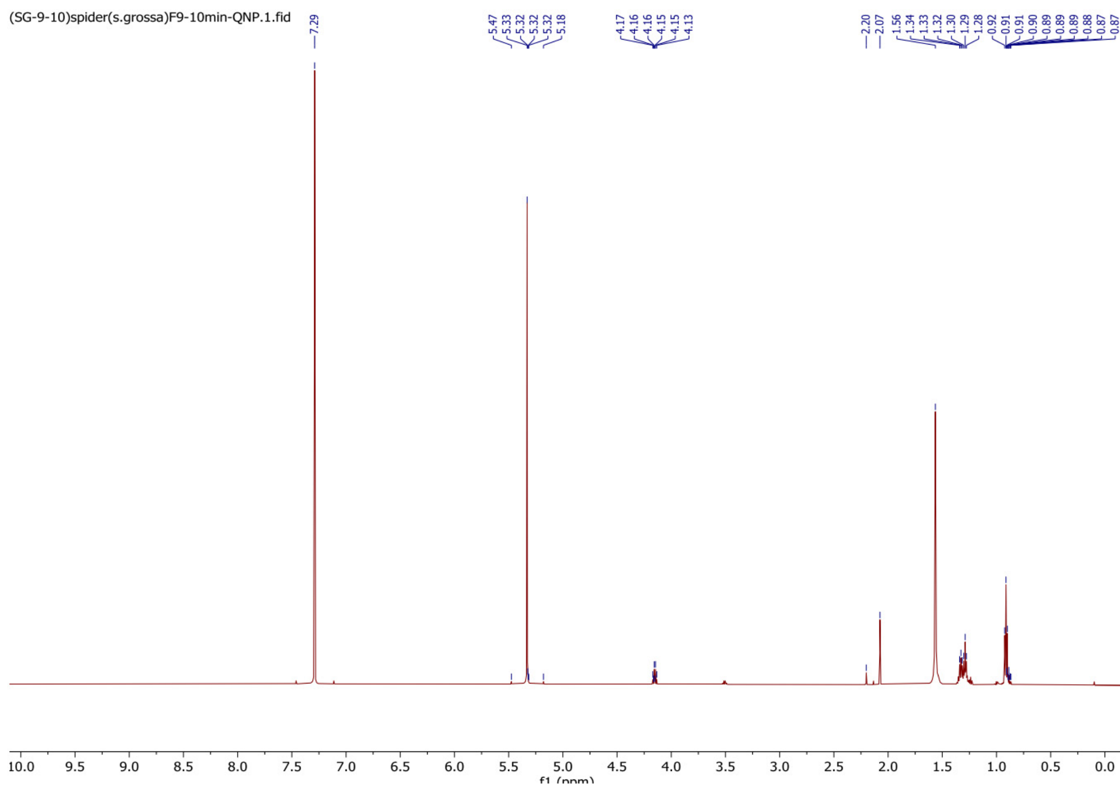


Figure A.2: **^{1}H NMR spectrum of *N*-4-methylpentanoyl-*O*-butyryl-L-serine produced by female *Steadoda grossa.*** The compound was extracted from webs of females, isolated by high performance liquid chromatography (see SFigure 1), and the ^{1}H NMR spectrum was recorded on a Bruker Avance 600 equipped with a QNP (600 MHz) using $CDCl_3$.

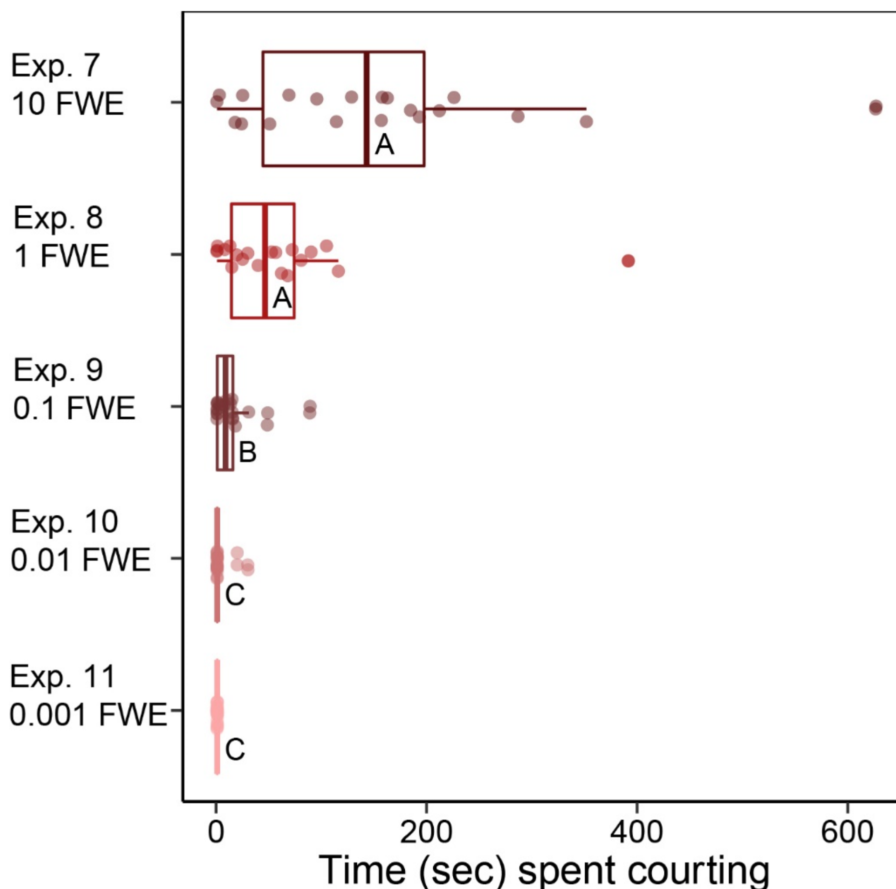


Figure A.3: **Effect of contact pheromone dose on the extent of courtship by *Steatoda grossa* males.** Time spent courting by *S. grossa* males in response to a ternary blend of synthetic contact pheromone components [*N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**12**); *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**16**); *N*-4-methylvaleroyl-*O*-hexanoyl-L-serine (**17**)] tested at five levels of female web equivalents (FWEs = amount of analyte present in the extract of a web from a single female *S. grossa*). Circles and boxplots show the time single male spiders courted in each replicate and the distribution of data (minimum, first quartile, median, third quartile, maximum), respectively. Medians with different letters indicate statistically significant differences in courtship responses; Kruskal-Wallis χ^2 test with Benjamini-Hochberg correction to account for multiple comparisons.

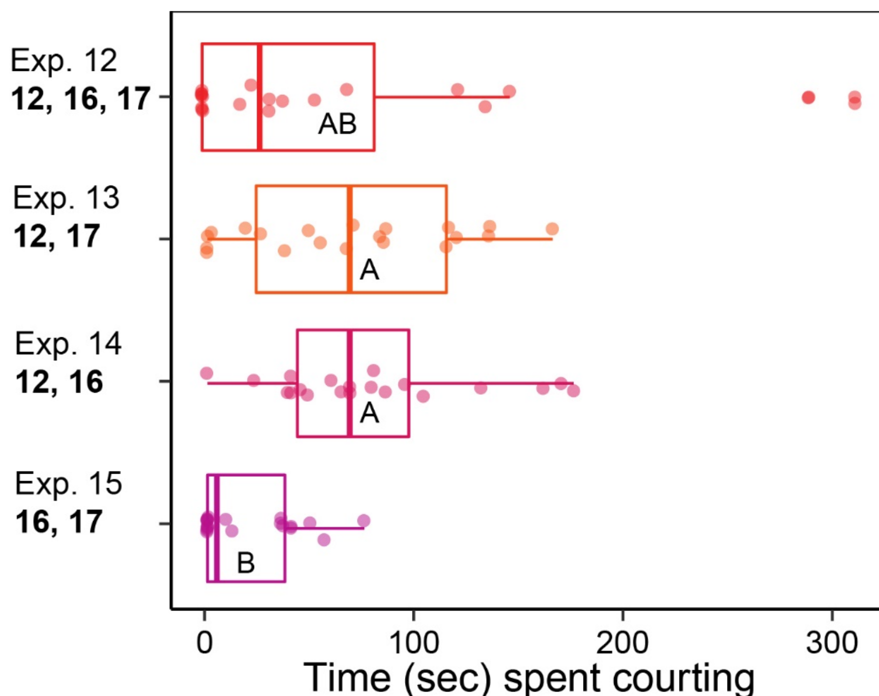


Figure A.4: **Effect of blend composition of contact pheromone components on the extent of courtship by *Steatoda grossa* males.** Time spent courting by *S. grossa* males in response to ternary and binary blends of synthetic contact pheromone components [*N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**12**); *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**16**); *N*-4-methylvaleroyl-*O*-hexanoyl-L-serine (**17**)] tested at one female web equivalents (amount of analyte present in the extract of a web from a single female *S. grossa*). Circles and boxplots show the time single male spiders courted in each replicate and the distribution of data (minimum, first quartile, median, third quartile, maximum), respectively. Medians with different letters indicate statistically significant differences in courtship responses; Kruskal-Wallis χ^2 test with Benjamini-Hochberg correction to account for multiple comparisons.

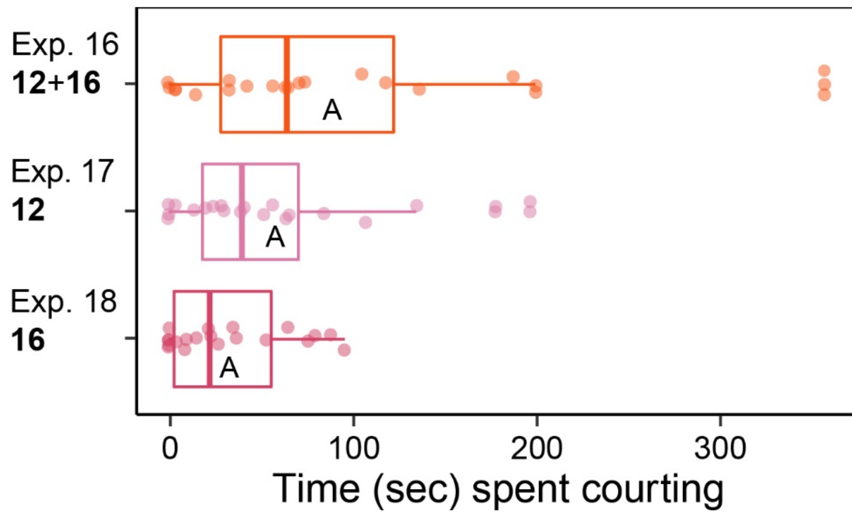


Figure A.5: **Effect of contact pheromone component(s) on the extent of courtship by *Steatoda grossa* males.** Time spent courting by *S. grossa* males in response to synthetic contact pheromone components [*N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**12**); *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**16**)] tested singly or in binary combination at one female web equivalent (amount of analyte present in the extract of a web from a single female *S. grossa*). Circles and boxplots show the time single male spiders courted in each replicate and the distribution of data (minimum, first quartile, median, third quartile, maximum), respectively. Medians with different letters indicate statistically significant differences in courtship responses; Kruskal-Wallis χ^2 test with Benjamini-Hochberg correction to account for multiple comparisons.

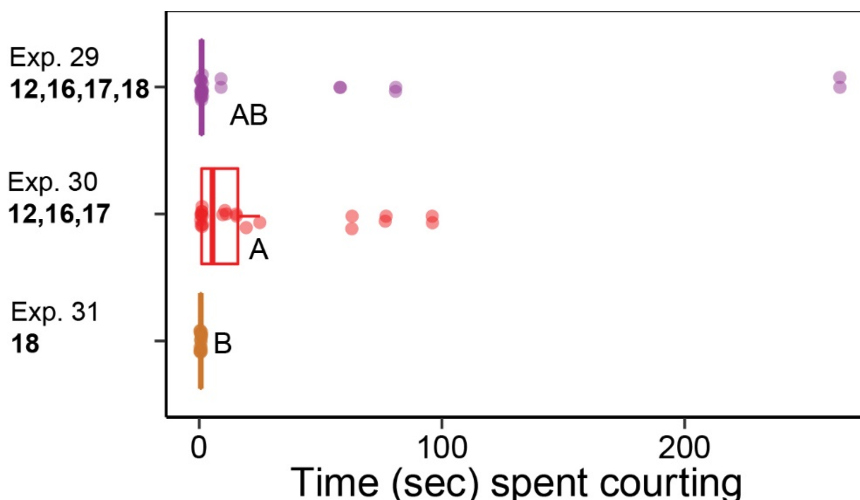


Figure A.6: **Effect of contact pheromone components and their breakdown product on the extent of courtship by *Steatoda grossa* males.** Time spent courting by *S. grossa* males in response to (i) the synthetic contact pheromone components [*N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**12**); *N*-4-methylvaleroyl-*O*-isobutyroyl-L-serine (**16**); *N*-4-methylvaleroyl-*O*-hexanoyl-L-serine (**17**)], (ii) their breakdown product *N*-4-methylvaleroyl-L-serine (**18**) and (iii) all combined (**12**, **16**, **17**, **18**), all stimuli tested at one female web equivalents (amount of analyte present in the extract of a web from a single female *S. grossa*). Circles and boxplots show the time single male spiders courted in each replicate and the distribution of data (minimum, first quartile, median, third quartile, maximum), respectively. Means with different letters indicate statistically significant differences in courtship responses; Kruskal-Wallis χ^2 test with Benjamini-Hochberg correction to account for multiple comparisons.

Syntheses

N-Boc-O-(*S*)-butyryl-L-serine benzyl ester

Butyric acid (3.38 mmol, 1 eq., 0.298 g) was added to a stirred mixture of N-boc-L-serine benzyl ester (3.38 mmol, 1 eq., 1.0 g) in dichloromethane (30 mL). After adding N,N'-dicyclohexylcarbodiimide (3.38 mmol, 1 eq., 0.696 g), followed by a catalytic amount of 4-(N,N-dimethylamino)pyridine, the reaction mixture was stirred 12 h at ambient temperature. Then it was purified by column chromatography with pentane/diethyl ether (2:1) as the eluent to give pure N-boc-O-(*S*)-butyryl-L-serine benzyl ester (1.1 g, 89 %). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ [ppm] = 7.36-7.30 (m, 5H), 5.33 (d, J = 8.5 Hz, 1H), 5.21 (d, J = 12.2 Hz, 1H), 5.13 (d, J = 12.2 Hz, 1H), 4.60 (dt, J = 8.2, 3.8 Hz, 1H), 4.47 (dd, J = 11.2, 4.0, 1H), 4.30 (dd, J = 11.2, 3.5, 1H), 2.17 (td, J = 7.4, 5.4, 2H), 1.55 (q, J = 7.5 Hz, 2H), 1.43 (s, 9H), 0.89 (t, J = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 173.0, 169.7, 155.1, 135.1, 128.6, 128.5, 128.4, 80.3, 67.5, 64.1, 53.1, 35.7, 28.3, 18.2, 13.6.

O-(*S*)-Butyryl-L-serine benzyl ester

Trifluoroacetic acid (10 mL) was added dropwise to a stirred solution of N-boc-O-(*S*)-butyryl-L-serine benzyl ester (1.0 g, 2.74 mmol) in dichloromethane (25 mL) at room temperature. The mixture was stirred 1.5 h, followed by *in vacuo* evaporation of the solvent and volatile constituents. The crude amino ester was characterized by NMR spectroscopy and used directly in the next step without purification. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ [ppm] = 7.37-7.30 (m, 5H), 5.26 (d, J = 12.0 Hz, 1H), 5.19 (d, J = 12.0 Hz, 1H), 4.56 (d, J = 3.6 Hz, 2H), 4.36 (t, J = 3.6, 1H), 2.25-2.12 (m, 2H), 1.53 (h, J = 7.4 Hz, 2H), 0.87 (t, J = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 173.3, 166.8, 134.0, 129.0, 128.7, 128.6, 68.9, 61.1, 52.8, 35.2, 17.9, 13.3.

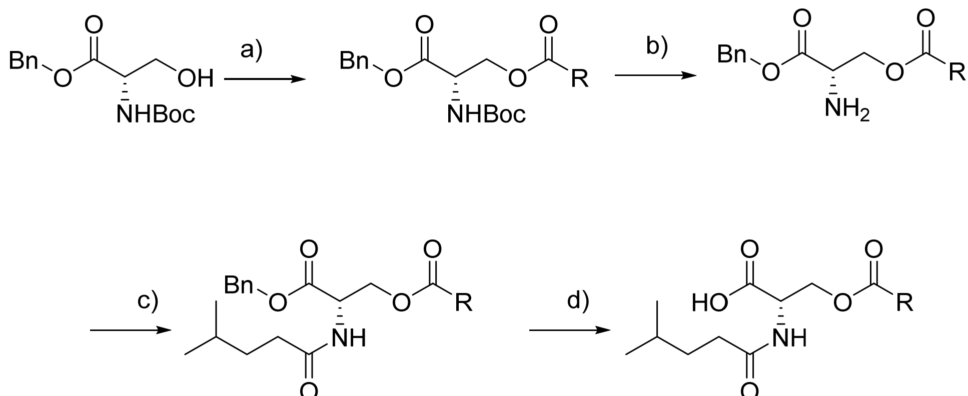
N-4-Methylpentyl-O-(*S*)-butyryl-L-serine benzyl ester

Triethylamine (7.92 mmol, 3.0 eq., 0.8 g) and 4-methylpentanoyl chloride (5.28 mmol, 2.0 eq. 0.71 g) were added dropwise under stirring and ice cooling to a solution of O-(*S*)-butyryl-L-serine benzyl ester (0.7 g, 2.64 mmol) in dichloromethane (20 mL). After stirring the resulting solution 2.5 h at room temperature, the mixture was washed with a saturated solution of $NaHCO_3$ and brine, dried over $MgSO_4$, and filtered. The final product was purified by column chromatography with a mixture of pentane and diethyl ether (1:2) to yield pure N-4-methylpentyl-O-(*S*)-butyryl-L-serine benzyl ester (0.57 g, 60%). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ [ppm] = 7.37-7.29 (m, 5H), 6.30 (d, J = 7.8 Hz, 1H), 5.17 (q, J = 12.0 HZ, 2H), 4.90 (dt, J = 7.6, 3.6 HZ, 1H), 4.48 (dd, J = 11.4, 4.0 Hz, 1H), 4.33 (dd, J = 11.4, 3.4, 1H), 2.26-2.21 (m, 2H), 2.16 (td, J = 7.0, 2.8 2H), 1.60-1.48 (m, 5H), 0.91-0.86 (m, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.1, 169.6, 135.0, 128.6, 128.6, 128.4, 67.6, 63.8, 51.8, 35.7, 34.4, 34.3, 27.7, 22.3, 22.3 18.2, 13.6.

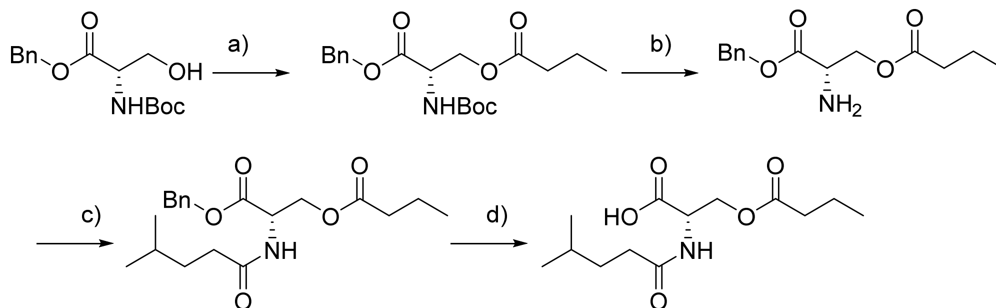
N-4-Methylpentyl-O-(*S*)-butyryl-L-serine

To a solution of N-4-methylpentyl-O-(*S*)-butyryl-L-serine benzyl ester (0.5 g, 1.37 mmol) in absolute ethanol (25 mL) was added 10% Pd-C catalyst (100 mg). After stirring the reaction mixture under H_2 at room temperature overnight, the catalyst was removed

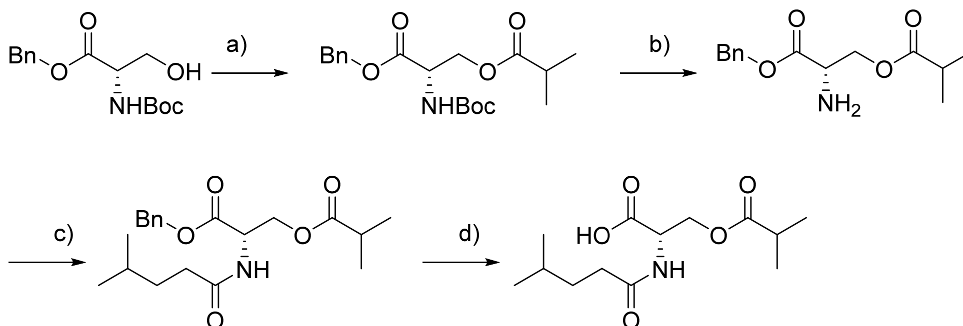
by filtration over celite. Concentration under reduced pressure gave N-4-methylpentyl-O-(*S*)-butyryl-L-serine (0.33 g, 90%). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ [ppm] = 8.42 (br, 2H),6.44 (d, J = 7.4 Hz, 1H), 4.85 (m, 1H), 4.52 (dd, J = 11.6, 4.4 Hz, 1H), 4.42 (dd, J = 11.6, 3.4, 1H), 2.29 (dt, J = 16.4, 7.6 4H), 1.69-1.50 (m, 3H), 0.94 (t, J = 7.4 Hz, 3H), 0.90 (d, J = 6.4 Hz, 6H). ^{13}C NMR (101 MHz, CDCl3) δ 174.5, 173.5, 171.6, 63.6, 51.9, 35.8, 34.4, 34.3, 27.7, 22.2, 22.9, 18.3, 13.5.



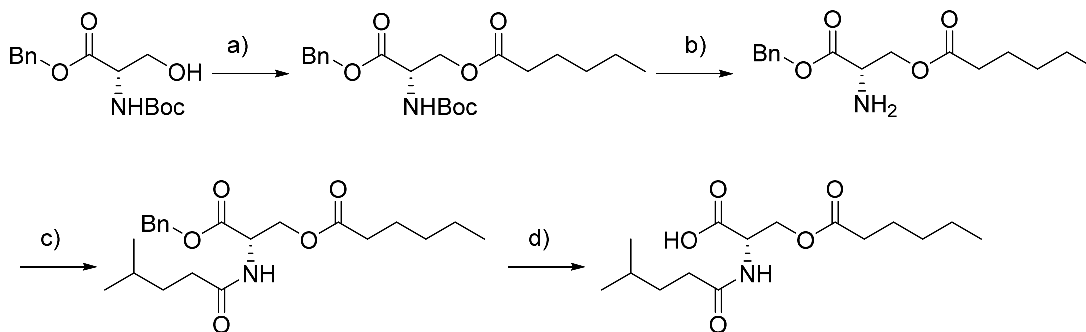
R = butyl, isobutyl or hexyl; a) butyric acid, isobutyric acid or hexanoic acid, N,N'-dicyclohexylcarbodiimide, 4-dimethylaminopyridine; b) trifluoroacetic acid; c) 4-methylpentanoyl chloride, triethylamine; d) Pd/C, hydrogen gas.



a) butyric acid, N,N'-dicyclohexylcarbodiimide,4-dimethylaminopyridine; b) trifluoroacetic acid;
c) 4-methylpentanoyl chloride, triethylamine; d) Pd/C, hydrogen gas.



a) isobutyric acid, N,N'-dicyclohexylcarbodiimide,4-dimethylaminopyridine; b) trifluoroacetic acid;
c) 4-methylpentanoyl chloride, triethylamine; d) Pd/C, hydrogen gas.



a) hexanoic acid, N,N'-dicyclohexylcarbodiimide,4-dimethylaminopyridine; b) trifluoroacetic acid;
c) 4-methylpentanoyl chloride, triethylamine; d) Pd/C, hydrogen gas.

N-Boc-O-(*S*)-isobutyryl-L-serine benzyl ester

^{1}H-NMR ($CDCl_3$, 500 MHz): δ [ppm] = 7.36-7.30 (m, 5H), 5.34 (d, J = 8.5 Hz, 1H), 5.20 (d, J = 12.2 Hz, 1H), 5.13 (d, J = 12.2 Hz, 1H), 4.60 (dt, J = 8.4, 4.0 Hz, 1H), 4.46 (dd, J = 11.2, 4.0, 1H), 4.29 (dd, J = 11.2, 3.5, 1H), 2.45 (p, J = 7.0, 1H), 1.43 (s, 9H), 1.17 (d, J = 7.0 Hz, H), 1.07 (dd, J = 10.4, 7.0 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 176.4, 169.7, 155.1, 135.1, 128.6, 128.5, 128.3, 127.9, 80.2, 67.5, 66.0, 64.1, 53.2, 34.0, 33.7, 28.3, 19.0, 18.9, 18.8.

N-Boc-O-(*S*)-hexyl-L-serine benzyl ester

^{1}H-NMR ($CDCl_3$, 500 MHz): δ [ppm] = 7.36-7.30 (m, 5H), 5.33 (d, J = 8.5 Hz, 1H), 5.24 (d, J = 12.2 Hz, 1H), 5.17 (d, J = 12.2 Hz, 1H), 4.63 (dt, J = 8.2, 3.8 Hz, 1H), 4.50 (dd, J = 11.2, 4.0, 1H), 4.33 (dd, J = 11.2, 3.5, 1H), 2.22 (q, J = 7.4, 2H), 1.57 (q, J = 7.5 Hz, 2H), 1.47 (s, 9H), 1.36-1.24 (m, 4H), 0.91 (t, J = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 173.3, 169.7, 155.1, 135.1, 128.6, 128.5, 128.3, 80.3, 67.5, 64.1, 53.1, 33.8, 31.2, 28.3, 24.4, 22.3, 13.9.

O-(*S*)-Isobutyryl-L-serine benzyl ester

^{1}H-NMR ($CDCl_3$, 500 MHz): δ [ppm] = 8.02 (br, 2H), 7.37-7.30 (m, 5H), 5.26 (d, J = 12.0 Hz, 1H), 5.19 (d, J = 12.0 Hz, 1H), 4.57 (dd, J = 3.6, 2.2 Hz, 2H), 4.39 (t, J = 3.6, 1H), 2.47 (p, J = 7.0 Hz, 1H), 1.19 (d, J = 7.0 Hz, 1H), 1.05 (dd, J = 9.8, 7.0 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 173.3, 166.7, 134.0, 129.0, 128.7, 128.6, 69.0, 61.1, 52.8, 35.2, 17.9, 13.3.

O-(S)-Hexyl -L-serine benzyl ester

^{1}H-NMR ($CDCl_3$, 500 MHz): δ [ppm] = 7.37-7.30 (m, 5H), 5.25 (d, J = 12.0 Hz, 1H), 5.19 (d, J = 12.0 Hz, 1H), 4.56 (d, J = 3.6 Hz, 2H), 4.33 (t, J = 3.6, 1H), 2.25-2.12 (m, 2H), 1.51 (p, J = 7.6 Hz, 2H),1.33- 1.18 (m, 4H) 0.87 (t, J = 7.2 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 173.4, 166.9, 134.1, 128.9, 128.7, 128.5, 68.8, 61.1, 52.7, 33.3, 31.1, 24.1, 22.2, 13.8.

N-4-Methylpentyl-O-(*S*)-isobutyryl-L-serine benzyl ester

^{1}H-NMR ($CDCl_3$, 500 MHz): δ [ppm] =11.04. (br, 1H), 7.40-7.30 (m, 5H), 6.52 (d, J = 7.6 Hz, 1H), 5.24-5.14 (m, 2H), 4.90 (dt, J = 7.6, 3.6 HZ, 1H), 4.49 (dd, J = 11.6, 4.0 Hz, 1H), 4.37 (dd, J = 11.6, 3.4, 1H), 2.45 (dq, J = 14.0, 7.0, 1H), 2.32-2.26 (m, 2H), 1.60-1.48 (m, 3H), 1.08 (dd, J = 7.0, 5.6 Hz, 6H), 0.89 (d, J = 6.4 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 176.8, 174.6, 169.2, 134.8, 128.7, 128.4, 67.8, 63.5, 52.3, 34.4, 33.8, 27.7, 22.2, 18.8, 18.7.

N-4-Methylpentyl-O-(*S*)-hexyl-L-serine benzyl ester

^{1}H-NMR ($CDCl_3$, 500 MHz): δ [ppm] =7.34-7.25 (m, 5H), 6.42 (d, J = 7.8 Hz, 1H), 5.19-5.08 (m, 2H), 4.87 (dt, J = 7.8, 3.8 HZ, 1H), 4.44 (dd, J = 11.4, 4.0 Hz, 1H), 4.30 (dd, J = 11.4, 3.6, 1H), 2.23-2.18 (m, 2H), 2.15 (td, J = 7.6, 3.0 2H), 1.56-1.45 (m, 5H), 1.30-1.17 (m, 4H) 0.87-0.82 (m, 9H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 173.2, 173.1, 169.5, 135.1, 128.6, 128.5, 128.3, 67.5, 63.7, 51.7, 34.3, 34.3, 33.8, 31.2, 27.7, 24.4, 22.3, 22.2, 22.2, 13.9.

N-4-Methylpentyl-O-(*S*)-isobutyryl-L-serine

^{1}H-NMR ($CDCl_3$, 500 MHz): δ [ppm] =10.20. (br, 1H), 6.56 (d, J = 7.0 Hz, 1H), 4.86 (q, J = 3.6 HZ, 1H), 4.47 (qd, J = 11.6, 3.4, 2H), 2.58 (dq, J = 14.0, 7.0, 1H), 2.32-2.26 (m, 2H), 1.60-1.48 (m, 3H), 1.17 (dd, J = 7.0, 2.6 Hz, 6H), 0.92 (d, J = 6.4 Hz, 6H). ^{13}C NMR

(101 MHz, $CDCl_3$): δ 177.1, 174.7, 171.8, 63.5, 52.2, 34.4, 34.3, 33.9, 27.7, 22.2, 22.2, 18.9.

N-4-Methylpentyl-O-(*S*)-hexyl-L-serine

^{1}H-NMR ($CDCl_3$, 500 MHz): δ [ppm] = 9.83 (br, 1H),6.49 (d, J = 7.4 Hz, 1H), 4.84 (m, 1H), 4.49 (dd, J = 11.6, 4.2 Hz, 1H), 4.42 (dd, J = 11.6, 3.4, 1H), 2.29 (dt, J = 18.8, 7.6 Hz, 4H), 1.57 (ddt, J = 37.6, 14.8, 7.4 Hz, 5H), 1.29 (tt, J = 8.4, 5.4 Hz, 4H), 0.89 (dd, J = 10.0, 6.4 Hz, 9H).

^{13}C NMR (126 MHz, CDCl3) δ 174.6, 173.7, 171.6, 63.6, 51.9, 34.3, 33.9, 31.2, 27.7, 24.4, 22.2, 22.2, 22.1, 13.8.

6-Methylheptanamide

6-Methylheptanoic acid (1 mmol) in dry DCM (3 mL), followed by dropwise addition of $SOCl_2$ (1.3 mmol, 0.094 mL), was added to a single-neck, round-bottom flask (10 mL) fitted with a reflux condenser and a calcium chloride-filled-guard tube. The reaction mixture was kept 6 h at 60 ºC and was then subjected to rotary evaporation under reduced pressure to remove solvent and excess $SOCl_2$, affording 6-methylheptanoyl chloride. The product was directly used in the next reaction step without further purification. The crude acid chloride was dissolved in THF and the mixture was added dropwise to aq NH_3 (14.8M, 2 mL) at 0 ºC. The mixture was allowed to warm to room temperature (rt), stirred overnight, and then diluted with DCM. The organic and aqueous layers were separated, and the aqueous layer was extracted twice with DCM. The combined organic layers were washed with brine, dried over Na_2SO_4, and filtered. The solvent was removed under reduced pressure to afford the crude amide which was purified via flash column chromatography on silica gel to yield 6-methylheptanamide (110 mg, 77%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.46 (s, 1H), 2.24 (t, *J* = 7.6 Hz, 2H), 1.63-1.57 (3H, m), 1.33 –1.24 (4H, m), 0.87 (d, *J* = 6.6 Hz, 6H).

Octanamide

Starting with octanoic acid and following the procedure described for 6-methylheptanamide, octanamide was obtained (113 mg, 79% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.47 (s, 2H), 2.24 (t, *J* = 7.6 Hz, 2H), 1.63 (h, *J* = 7.2 Hz, 2H), 1.36 – 1.24 (m, 8H), 0.92 – 0.83 (m, 3H).

4,6-Dimethylheptanamide

To a solution of 6-methyl-4-methyleneheptanoic acid (0.78 g, 5.0 mmol) in anhydrous MeOH (50 mL), 10% Pd/C (250 mg) was added in one portion. The black slurry was stirred under H_2 atmosphere (balloon) overnight before being filtered through celite. The filtrate was concentrated to give 4,6-dimethylheptanoic acid as a light yellow oil (Chen et al., 2017).

Starting with 4,6-dimethylheptanoic acid and following the procedure described for 6-methylheptanamide, 4,6-dimethylheptanamide was obtained (108 mg, 69% yield). ^{1}H

NMR (500 MHz, $CDCl_3$) δ 2.30 -2.18 (m, 2H), 1.70 - 1.62 (m, 2H), 1.58-1.49 (m, 1H), 1.48 - 1.40 (m, 1H), 1.15 - 1.09(m, 1H), 1.06 - 0.97 (m, 1H), 0.87 (dd, J = 6.6, 1.5 Hz, 6H), 0.84 (d, J = 6.6 Hz, 3H).

Methyl (4-methylpentanoyl)serinate

4-Methylpentanoic acid (Sigma-Aldrich) (1 mmol, 1 eq., 116 mg) was added to a stirred mixture of N-methyl-serinate (Sigma-Aldrich) (1 mmol, 1 eq., 119 mg) in dichloromethane (10 mL). N,N'-dicyclohexylcarbodiimide (1 mmol, 1 eq., 206 mg) was added followed by triethylamine (1 mmol, 1 eq., 101 mg). The reaction mixture was stirred 12 h at ambient temperature. The reaction product was purified by column chromatography with pentane/diethyl ether (2/1) as eluent to give methyl (4-methylpentanoyl)serinate (173 mg, 80 %).

4-Methylpentanoyl)serine (= *N*-4-Methylvaleroyl-L-serine)

To a solution of methyl (4-methylpentanoyl)serinate (173 mg, 0.8 mmol) in MeOH (3 mL) was added a solution of LiOH (96 mg, 4 mmol) in H_2O (1 mL) at 0 °C. After stirring 2 h at ambient temperature, the reaction was quenched with concentrated aqueous HCl. The mixture was extracted with AcOEt, and the organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography on silica gel with hexane/EtOAc (4/1) as eluent to give (4-methylpentanoyl)serine (146 mg, 90% yield).

Reference

Chen D-F, Chu JCK, and Rovis T (2017) Directed γ-C(sp^3)–H alkylation of carboxylic acid derivatives through visible light photoredox catalysis. *Journal of the American Chemical Society 139* (42), 14897-14900. DOI: 10.1021/jacs.7b09306