Female web-building spiders disperse 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.
Attracting or finding a mate is essential for all sexually reproductive animal species. The process is mediated by long-range communication signals that have chemical, auditory, visual, vibratory or multi-modal characteristics. Chemicals such as pheromones are deemed the oldest form of (sexual) communication signals and have evolved in various animal taxa, including mammals, myriapods, crustaceans and insects. Airborne pheromones have signal functions in the context of aggregation, territorial marking, warning, nest defence and reproduction. Volatile sex pheromones attract prospective mates, whereas cuticle-bound mate recognition pheromones impart reproductive isolation and insect speciation.

Sex pheromones have been most extensively studied in insects. Beetles, moths, ants and wasps all produce, and release, pheromones from specific glands located in various parts of their body. Many insects can actively time their pheromone production and release, and modulate the amount of pheromone they emit. Pheromones are perceived by olfactory receptors on the insects’ antennae, involving complex molecular interactions between pheromone receptors and the pheromone ligands. More than 3000 insect pheromones have already been identified. Using the insects’ antennae as an analytical tool to help locate candidate pheromone components in complex analytical samples has been instrumental in identifying many of these pheromones, particularly those that occur at trace quantities. 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.

Web-building spiders are multi-modal communicators, using primarily pheromonal and vibratory communication signals. Pheromones play major roles during habitat selection, mate competition, courtship and mate choice. Unlike insects that typically disseminate pheromones from specific gland tissues, female spiders deposit pheromones on their silken webs. Their webs attract males over long distances and, upon contact, elicit courtship in males, implying the release of mate attractant pheromone components from the web and the presence of contact pheromone components on the web. 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 and the widow spiders Latrodectus hasselti and L. hesperus. Female L. triangularis deposit (R)-3-[(R)-3-hydroxybutyrlyloxy]-butyric acid on their webs, whereas female L. hasselti and L. hesperus deposit serine derivatives [N-3-methyl-butyrol-0-(S)-2-methylbutyryl-1-serine methyl ester, N-3-methylbutanoyl-O-methylpropanoyl-1-serine methyl ester] (Fig. 1a). Both I and its breakdown monomer, (R)-3-hydroxybutyric acid, induce courtship by male L. triangularis. 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. As enzyme activity is pH-dependent, and spider females might be able to adjust their silk’s pH, 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) and Steatoda grossa inhabits predominantly buildings, where it reproduces year-round irrespective of season. As Steatoda and Latrodectus spiders are close phylogenetic relatives, 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.

**Results and discussion**

**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 three days to build their webs on a prism scaffold (Fig. 1b), building upon previous results that only mature females produce pheromone components. We then methanol-extracted pooled webs from each of the two female groups and analysed extracts by gas chromatography–mass spectrometry (GC-MS). These analyses revealed seven compounds (5–11 in Fig. 1d; pyrrolidin-2-one (5), 4-hydroxyhydrofuran-2(3H)-one (6), nonanoic acid (7), dodecanoic acid (8), 6-methylheptanamide (9), octanamide (10), 4,6-dimethyl heptanamide (11), S-Table 1) 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. 1c) with a synthetic blend of 5–11 (Exp. 1), or with web extract (positive control; Exp. 2), and the corresponding filter paper with solvent control. As only web extract, but not the blend of 5–11, elicited courtship by males (N = N = 20, W = 370, P = 0.001, Exp. 1 + 2, Fig. 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 (Supplementary Fig. 2) were then analysed by HPLC-tandem mass spectrometry (MS/MS) and by nuclear magnetic resonance (1H NMR) spectroscopy. HPLC-MS/MS analyses revealed an unknown compound (12) with a molecular formula of C_{13}H_{23}NO_3 and fragmentation ions 186, 274 (M + 1) and 296 (M + Na), indicating a molecular weight of 273 (Fig. 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. 1a). Yet, the 1H NMR spectrum of unknown 12 (Supplementary Fig. 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 and reanalysed aliquots of this extract by GC-MS. These analyses revealed not only one, but three serine methyl ester derivatives (Fig. 2b; N-4-methylvaleryl-O-butryroyl-1-serine methyl ester (13), N-4-methylvaleryl-O-isobutyroyl-1-serine methyl ester (14), and N-4-methylvaleryl-O-hexanoyl-1-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. 2a) was also present in serine methyl esters 2 and 3 produced by L. hasselti and L. hesperus (Fig. 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. 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. 2b).

All three serine methyl ester derivatives had similar mass spectra (Fig. 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. 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 synthesised multiple standards (see Supplementary material Methods: Synthesises). Of these, N-4-methylvaleroyl-O-isobutyryl-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-isobutyryl-L-serine (12); N-4-methylvaleroyl-O-isobutyryl-L-serine (16); N-4-methylvaleroyl-O-hexanoyl-L-serine (17); Fig. 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. 1c) bioassays, a ternary blend of the synthetic acids 12, 16 and 17, and tested at one web equivalent, elicited courtship behaviour by S. grossa males comparable to web extract (Exp. 3 vs. Exp. 4: N1 = N2 = 20, Z = −0.39, P = 0.521, Fig. 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. 1d) did not enhance the courtship behaviour on their own (Exp. 6, Fig. 2d). In contrast, the ternary acid blend induced courtship behaviour in a dose-dependent manner (Exps. 7–11: $\chi^2 = 61.75$, df = 4, $P < 0.001$; Supplementary Fig. 3). Binary blends of the acids also induced courtship behaviour, but their effect differed according to blend constituents (Exps. 12–15: $\chi^2 = 11.19$, df = 3, $P = 0.010$; Supplementary Fig. 4). Acids 12 and 16 tested singly elicited courtship as effectively as in binary combination (Exps. 16–18: $\chi^2 = 3.65$, df = 2, $P = 0.160$; Supplementary Fig. 5).

**Origin of contact pheromone components.** Silk glands have been hypothesised, 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. 2a), we proceeded to trace its origin. For all analyses, we cold-euthanized...
spiders, extracted body tissue in a methanol/saline solution, centrifuged extracts, and analysed aliquots of each tagma or tissue sample by HPLC-MS for the presence and 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 and 16 (Exp. 19: \( N = 22, W = 21, P = 0.004 \), Fig. 3a), we then screened abdominal haemolymph and five specific abdominal tissues, including all eight silk glands combined, for the presence of 12 and 16. With only silk gland samples containing 12 and 16 (Exp. 20: \( N = 20, \chi^2 = 70.96, df = 6, P < 0.001 \), Fig. 3b), we analysed glands separately and found that it was the posterior...
aggregate gland that exclusively, or most abundantly, contained 12 and 16 (Exp. 21: \(N = 30, \chi^2 = 36.00, \text{df} = 6, P < 0.001\), Fig. 3c). Although not specifically tested, it is likely that the posterior aggregate gland also produces contact pheromone component 17.

**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\(^5^6\), using web extract (instead of webs) as the test stimulus (Fig. 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. 4b). However, when offered a choice between the blend of volatile compounds 5–11 unique to sexually mature females (Fig. 1d) and a solvent control, males exhibited no attraction responses (Exp. 23: \(N = 20 P = 0.588\), Fig. 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\(^3^1\,^4^2\) that the dimer contact pheromone 1 of the spider *L. triangularis* breaks down to a volatile monomer attractant (4) (Fig. 1a), we
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 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 one male, confirming the mating attractant pheromone function of the carboxylic acids (Exp. 28: *N* = 10, *P* = 0.011, Fig. 4f).

hypothesised (Fig. 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-methylvaleryl-L-serine (18) and three corresponding carboxylic acids [butyric (19), isobutyric (20), hexanoic (21)], and that these volatile acids then attract males. Realising 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. 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*<sub>0 days</sub> = *N*<sub>14 days</sub> = 70, *P* < 0.001, Fig. 4d). Moreover, our attraction hypothesis was supported by Y-tube olfactometer data (Fig. 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: *χ*<sup>2</sup> = 12.78, df = 2, *P* < 0.001, Supplementary Fig. 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 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 one male, confirming the mating attractant pheromone function of the carboxylic acids (Exp. 28: *N* = 10, *P* = 0.011, Fig. 4f).
Mechanisms underlying the transition of contact pheromone components to mate attractant pheromone components. We hypothesised 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 metre57. 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_16,89 = 108.44, p < 0.001; Fig. 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. 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 webs45, 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 relatives41, we predicted that they produce not only similar serine-derived contact pheromone components9 (see 3 and 12) but also a similar or identical CEH to hydrolyse them. To test our prediction, we extracted webs in Sørensen buffer58 from three groups of spiders: (1) adult virgin female L. hesperus (positive control, known to have a CEH45); (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 ten 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. 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 web30 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 tenfold 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. grossa43. Female L. hesperus also produce a serine derivative contact pheromone component (Fig. 1a) that is likely hydrolysed by a carboxyl ester hydrolase, reported to be present on L. hesperus webs44.

Sustained dissemination of mate attractive 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 release59, thus prolonging the attractiveness of pheromonal markings60.

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-dependent46, and spiders lower the pH in their spinning apparatus to convert aqueous silk to solid silk threads47,61,62. 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 dissemination63, and to modulate the amount of pheromone they emit64. 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—that their pheromones originate from the same silk gland. Pheromone-producing glands often contain a sufficient large amount of pheromone anlyte for structural elucidation. 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. 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, possibly, many more constituents.

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 an incentive for comparative studies in other spider taxa.

Methods

Experimental spiders. Experimental spiders were maintained as previously reported52. 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 mm x 20 mm) and provisioned with the vinegar flies Drosophila melanogaster. Subadult 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).

Identification of contact pheromone components: Preparation of web extracts (summer 2017; spring and summer 2018). Each of the 100 spiders was allowed to build her web for three days on a wooden triangular prism scaffold (30 cm x 25 cm x 22 cm)44 of bamboo skewers (GoodCook, CA, USA) (Fig. 1b). After the spiders were removed from the scaffold, their webs were reeled up with a glass rod (10 cm x 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 vial was extracted for 24 h at room temperature. Prior to extraction, the silk was removed and the sample was concentrated under a steady nitrogen stream to the desired concentration.

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 5977 A MSD, both fitted with a DB-5 GC-MS column (30 m x 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 cm3 min–1.

Identification of contact pheromone components: high-performance liquid chromatography (HPLC) of web extracts. Web extract of virgin 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 C18 column (250 mm x 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. 1c) for the presence of contact pheromone components. All eight fractions that elicited courtship responses by males (Supplementary Fig. 1) were analysed by HPLC-tandem MS/MS.

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 spurious C18 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-QqTOF) 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 nebuliser was set to 4 bar and the capillary voltage to 4200 V. The column was eluted with a 0.4 µL/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.

Identification of contact pheromone components: 1H NMR analyses of a bioactive fraction. In HPLC-MS analyses, a single bioactive fraction (9–10 min) appeared to contain only a single compound. This fraction was then further investigated using 1H NMR spectroscopy. The 1H NMR spectrum was recorded on a Bruker Advance 600 equipped with a QNP (900 MHz) using CDCl3. Signal positions (δ) are given in parts per million from tetramethylsilane (δ 0) and were measured relative to the signal of the solvent (δ H NMR: CDCl3; δ 7.26).

Identification of contact pheromone components: synthesis of candidate pheromone components. The synthesis of candidate pheromone components and synthetic intermediates are reported in the SI.
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, 42] 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 the responses of spiders. The sample size for each experiment was set to 20 unless otherwise stated.

**Identification of contact pheromone components: T-rod bioassays (specific experiments) (fall 2017; spring and summer 2018).** Experiment 1 (fall 2017) tested a synthetic blend of volatile compounds 5-11 unique to mature S. grossa females (Fig. 1c and Supplementary Table 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 (Exp. 4), the same ternary blend (12, 16 and 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 FWE: 0.001 (Exp. 7); 0.01 (Exp. 8); 0.1 (Exp. 9); 1.0 (Exp. 10); and 10 (Exp. 11).

Parallel experiments 12-15 (with 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 (Exp. 17, 18).

**Origin of contact pheromone components (fall 2020).** To trace the origin of contact pheromone component 12 (and coeluroid 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 MgCl2, 4 mM NaHCO3, 4 mM CaCl2, 200 mM glucose, pH 7.4). Sampled were harvested homogenized (Polytron (20 mm Pellet Pestle Microtube, 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, Weihingen, DE) to obtain the supernatant for HPLC-MS analyses (see above) for the presence of 12 and 16. Three sequential sets of dissections aimed to determine (1) the 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 and 16, whereas only one of 11 thorax samples contained 12 and 16 (Exp. 19), albeit at only trace amounts. With 12 and 16 being present in the abdomen, 20 additional abdomens were dissected[68] to obtain separate samples of (i) haemolymph (25 µL), (ii) ventral cuticle (~0.5 cm² near the pedicel, (iii) all silk glands combined, and (iv) the gut (with anus, coacala and Malpighian tubules). The 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 and 16 (Exp. 20). Having established that only silk gland samples contained 12 and 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) pyriform gland. The glands from three spiders were combined in each sample and extracted in 30 µL methanol. Seven of ten posterior aggregate gland samples contained 12 and 16, with other silk gland samples not containing 12 and 16 or in only trace amounts (Exp. 21).

**Transition of contact pheromone components to mate attractant pheromone components: evidence for hydrolysis of contact pheromone components (12, 16 and 17) (spring 2021).** To test for the hydrolysis of the contact pheromone components 12, 16, and 17, we compared their breakdown rates (18) (12 + 16 + 17 = 18) in 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 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 and 50 ng/µL as external standards.

**Transition of contact pheromone components to mate attractant pheromone components: Y-tube olfactometer bioassays (specific experiments) (summer 2018).** In experiments 22, 23 and 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. 1d) (Exp. 23, N = 24), (iii) all breakdown products of the contact pheromone components 12, 16 and 17, consisting of the amide N-4-methylvaleryl-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 (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) used as an internal standard. Per web equivalent, there were 30 19, 3 ng of 20 and 54 ng of 21. The amide 18 was presented at 200 ng per web equivalent, as determined using N-3-methylbutanoyl-L-serine methyl ester as an external standard.

**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 ten 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 (Anachem, Ontario, Canada, 2.8 µg of 19, 0.112 µg of 20 and 1.52 µg of 21), whereas the control trap received mineral oil only. Test stimuli were disseminated from a 400-µL microcentrifuge tube (Evergreen Scientific, Rancho Dominguez, CA, USA) with a hole in its lid punctured by a No. 3 insect pin (Hamilton Bell, Montvale, NJ, USA). Every week for 4 months (September to December 2018), traps were checked, lures were replaced, and the position of the treatment and the control trap within each trap pair was re-randomised.

**Mechanisms underlying the transition of contact pheromone components to mate attractant pheromone components: testing for pH-dependent saponification of contact pheromone components (12, 16 and 17) (summer 2021).** To test whether pH alone catalyses saponification of the ester bond of contact pheromone components (12, 16 and 17), synthetic 12 was added to a 40% aqueous pH 7 buffer (pH 6.5; 200 µL mixture containing water and 200 µL of 1 N sodium carbonate (BD), pH adjusted to 7.0 with 1 N hydrochloric acid (BD) and used as an external standard) and the pH dependent breakdown of 12 over time was assessed by analysing (HPLC-MS).

**Mechanisms underlying the transition of contact pheromone components to mate attractant pheromone components: testing for pH-dependent saponification of contact pheromone components (12, 16 and 17) (summer 2021).** To test whether pH alone catalyses saponification of the ester bond of contact pheromone components (12, 16 and 17), synthetic 12 was added to a 40% aqueous pH 7 buffer (pH 6.5; 200 µL mixture containing water and 200 µL of 1 N sodium carbonate (BD), pH adjusted to 7.0 with 1 N hydrochloric acid (BD) and used as an external standard) and the pH dependent breakdown of 12 over time was assessed by analysing (HPLC-MS).
Mechanisms underlying the transition of contact pheromone components to mate attractive pheromone components: testing for the presence of a carboxysteroldehydrolase (CEH) (summer 2021). To test for the presence of a carboxysteroldehydrolase (CEH), for each of three replicates we extracted (i) five webs of adult virgin female L. hesperus (positive control, known to have a CEH)\(^{(2)}\), (ii) 20 webs of subadult S. grossa (deemed to have not yet produced a CEH) and (iii) ten webs of adult virgin male 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\(^{(2)}\) and analysed by Bioinformatics Solutions (Waterloo, ON, CA). After web samples were incubated for 20 min at 60 °C in 2× sample volumes of 10% SDS (lauryl sulfate; protein-denaturing anionic detergent), they were sonicated for 400–1600 m/s. Peptides were eluted at a rate of 0.2 µm L/min and separated using a 5 µm particle size; Thermo Fisher) at a constant 523 m/z + 60 min: 4% formic acid; 55 m/z – 60 min: 4% formic acid; 55 m/z – 60 min: 4% formic acid.

Statistics and reproducibility. Data (Supplementary Table 2) were analysed statistically using R\(^{(2)}\). 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 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 and 25–27, as well as the hallway experiment 28 (revealing attraction of male spiders to volatile pheromone components) were analysed using an one-tailed\(^{(2)}\) binomial test, anticipating attraction of spiders to volatile mate attractive 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 generalised 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.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data, including raw data underlying the figures, can be found in Supplementary Data 1.

Code availability. All codes that were used to analyse the data can be found in Supplementary Data 1.
