The fatty acid elongase Bond is essential for Drosophila sex pheromone synthesis and male fertility

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Insects use a spectacular variety of chemical signals to guide their social behaviours. How such chemical diversity arises is a long-standing problem in evolutionary biology. Here we describe the contribution of the fatty acid elongase Bond to both pheromone diversity and male fertility in Drosophila. Genetic manipulation and mass spectrometry analysis reveal that the loss of bond eliminates the male sex pheromone (3R,11Z,19Z)-3-acetoxy-11,19-octacosa-

dien-1-ol (CH503). Unexpectedly, silencing bond expression severely suppresses male fertility and the fertility of conspecific rivals. These deficits are rescued on ectopic expression of bond in the male reproductive system. A comparative analysis across six Drosophila species shows that the gain of a novel transcription initiation site is correlated with bond expression in the ejaculatory bulb, a primary site of male pheromone production. Taken together, these results indicate that modification of cis-regulatory elements and subsequent changes in gene expression pattern is one mechanism by which pheromone diversity arises.
Sexual recognition in many insects is largely influenced by lipids on the cuticles, many of which function as pheromones. In numerous species, changes in the pheromone profile have been implicated as a mechanism underlying reproductive isolation and speciation. For example, in Lepidoptera and Drosophila, allelic variations in genes encoding pheromone biosynthesis enzymes result in different sex pheromone blends and assortative mating. However, much remains unknown about the molecular mechanisms underlying the evolution of pheromone profiles.

The identification of pheromone biosynthesis enzymes is a crucial step for understanding how pheromone signals evolve. In Drosophila, the oenocytes and ejaculatory bulb (EB) are the major sites of pheromone synthesis. In the oenocytes of Drosophila melanogaster, the elongation of female dienes is carried out by EloF (ref. 8). The desaturases Desat1, Desat2 and DesatF place double bonds at distinct positions in the hydrocarbon backbone of the female aphrodisiacs (7Z,11Z)-heptacosadiene and (7Z,11Z)-nonacosadiene. Desat1 also contributes to the generation of characteristic male monoenes (7Z)-tricosene, and (7Z)-pentacosene. Modification of the enhancer regions of DesatF is thought to underlie differences in the species and sex-specific pheromone profiles of various Drosophila species.

In comparison, the mechanisms underlying pheromone synthesis in the EB are relatively sparse. Closely related drosophilid species exhibit large variations in the EB pheromone composition. Thus, the EB serves as an excellent model for understanding the evolution of pheromone diversity. The EB is connected to the male reproductive organs and its contents are secreted exogenously to the anogenital region. In D. melanogaster, two male sex pheromones, 11-cis-vaccenyl acetate (cVA) and (3R,11Z,19Z)-3-acetoxy-11,19-octacosadien-1-ol (CH503), are produced in the EB. Both molecules are transferred to females during courtship and function as anti-aphrodisiacs that suppress attraction from other males. To date, only one enzyme in the EB, Elo68a, has been shown to contribute to the synthesis of pheromones.

In this study, we sought to identify enzymes involved in the biosynthesis of pheromones in the EB in an effort to understand the evolution of pheromone signals. We show that jamesbond (also known as bond), which encodes a very-long-chain fatty acid (VLCPA) elongase, is expressed in the EB and male reproductive organs and plays an essential role in the synthesis of the male sex pheromone CH503 and male fertility. Loss of bond expression results in a significant decrease in male fertility. Surprisingly, Bond also regulates the fertility of conspecific male rivals and this effect is mediated partly by CH503. A comparative analysis across species of Drosophila shows that the gain of a novel transcription initiation site is correlated with a gain of bond expression in the EB, thus enabling the production of more complex pheromone mixtures.

**Results**

**Bond is essential for the synthesis of a sex pheromone.** The elongase Bond was identified as part of a RNA interference (RNAi) screen to discover genes that contribute to the biosynthesis of pheromones in the EB. The Drosophila GAL4-UAS transgene expression system was used to perform RNAi-mediated knockdown in a tissue-specific manner. Using the NP4004-GAL4 driver, which expresses in the male reproductive organs, EB, gut and parts of the central nervous system (Fig. 1a; Supplementary Fig. 1), the expression of candidate genes was silenced specifically in these organs and the lipid profiles assessed with ultraviolet laser desorption ionization mass spectrometry (UV-LDI MS). UV-LDI MS is a recently introduced method for cuticular lipid analysis that provides spatially resolved chemical profiles of intact insects.

In wild-type males, the mass spectral profiles of the anogenital region (Fig. 1b) and dissected EB (Supplementary Fig. 2) consist primarily of two signals corresponding to cVA and CH503. In contrast, direct mass spectrometry analysis of dissected testes and accessory glands showed no detectable signal for cVA or CH503 (Supplementary Fig. 2), indicating that the pheromones are produced primarily in the EB. Following RNAi-mediated knockdown of bond, the signal for CH503 was no longer detected (Fig. 1b). Since the NP4004 driver could represent the expression pattern of several genes proximal to bond, we generated a GAL4 line targeting the bond enhancer region. bond-GAL4 expression is evident in parts of the gut, testes and EB (Supplementary Fig. 1). Silencing bond expression with the bond driver also eliminated the signal for CH503 (Fig. 1b). The loss of CH503 in the EB was also observed when bond transcription is interrupted in a PBac insertion line, bondBPac. Notably, the defect was upon excision of the PBac insertion (bondBPac) or overexpression of bond in the bondBPac background (Fig. 1b).

To test whether the loss of CH503 signal could be due to a defect in secretion rather than production, we extracted lipids from pooled, dissected EBs of bondBPac, bondBPac, and a control PBac line, which has the same genetic background. Analysis by thin-layer chromatography did not reveal a notable signal for CH503 in extract from bondBPac flies, thus indicating a failure of CH503 synthesis rather than secretion (Fig. 1c). Quantitation by gas chromatography mass spectrometry (GCMS) showed a slight but non-significant decrease in cVA levels in bondBPac flies when compared with the background control line (Supplementary Fig. 2). Interestingly, both UV-LDI MS and GCMS analysis of bondBPac extract revealed a prominent signal at m/z 377.27 ([M + K]⁺; Fig. 1d) that was found only in low quantities in bondBPac extract. On the basis of the electron impact spectra and exact mass measurements, the molecule is predicted to contain an acetate and to have the elemental composition C₂₄H₄₃O₂. We hypothesize that the compound is used for the synthesis of CH503 and, in the absence of bond, remains unincorporated. Taken together, these results indicate that bond expression in the EB is necessary for CH503 production but not cVA synthesis.

**Bond contributes to male fertility and male rivalry effects.** The product of the bond gene was previously described as a VLCPA elongase that is necessary for cytokinesis of Drosophila spermatocytes. When tested for fertility defects, both bondBPac males (1.33 ± 1.24 progeny per fly) and males in which bond expression is reduced using RNAi (bond > bondRNAi, 15.03 ± 12.46 progeny per fly) showed low fertility (Fig. 2a). Since bond contributes to both spermatogenesis and male pheromone production, we tested the possibility that CH503 may be used as a fertility cue by females. Given a choice between control bondBPac males and males of the same genotype perfumed with either 83 or 830 ng of CH503, females did not exhibit a statistically significant preference for the perfumed fly. However, there was a medium-sized effect (Pearson’s r = 0.26) resulting from the 83 ng per fly treatment compared with a small-sized effect (Pearson’s r = 0.06) resulting from the 830 ng per fly treatment (Fig. 2b). These results indicate that CH503 at an optimal dose modulates female preference.

Male fertility can also be influenced by social conditions. Previous studies have shown that across numerous taxa, males alter their mating behaviour and sperm allotment in response to rival males. In Drosophila, a combination of sensory cues underlies the rivalry effect including visual and chemical signals. To test whether bond and its biosynthetic products...
levels of CH503 in bonde03675 extract from bondres and production in the mutant background. Each mass spectrum was recorded from a single fly. (Fig. 3b,c). In contrast, the two orders of magnitude greater than the other transcripts (Fig. 3b,c). In contrast, the two orders of magnitude greater than the other transcripts. (Fig. 3b,c). In contrast, the two orders of magnitude greater than the other transcripts.

Figure 1 | Disruption of bond in the ejaculatory bulb results in loss of the sex pheromone CH503. (a) Confocal image of NP4004->mCD8::GFP shows broad expression in the male reproductive organs (arrowheads), scale bar, 100 μm. (b) UV-LDI mass spectra recorded from the anogenital region of male flies. Signals corresponding to the mass-to-charge ratio (m/z) of cVA (m/z 349.24) and CH503 (m/z 503.38) are observed in NP4004 -> GCaMP (control) and bondRNAi males. The CH503 signal is not detected in NP4004 -> bondRNAi, bond> bondRNAi and bondRNAi males. Overexpression of bond rescued CH503 production in the mutant background. Each mass spectrum was recorded from a single fly. (c) Quantification of CH503 by HPTLC of EB extracts reveals low levels of CH503 in bondRNAi males compared with bondRNAi and control line 970 (m/z 503.38). Bars represent the mean of three replicates ± s.e.m. Each replicate consists of extract from 20 EBs. ANOVA with a Tukey’s multiple comparison test, *P < 0.05, **P < 0.001, ****P < 0.0001. (d) UV-LDI MS spectra of EB extract from bondRNAi and bondRNAi flies. The signal at m/z 377.27 ([M + K]+; green arrow) is detected with 13-fold higher intensity in extract from bondRNAi compared with bondRNAi, ANOVA, analysis of variance.

Contribute to rivalry-induced modulation of fertility, we exposed males from wild-type strains CantonS or Berlin to three rival males of the same genotype before mating (Fig. 2c). Consistent with previous work, mating duration increased with prior exposure to rivals, regardless of rival genotype (Fig. 2d). Surprisingly, males of both WT strains exposed to bondRNAi males produced fewer viable offspring when exposed to rivals with functional bond (CantonS and bondRNAi males; Fig. 2e). The decreased fertility was not the result of a shorter mating duration since copulation time was not different between each genotype in the rival-exposure conditions. Fertility increased slightly by exposing CantonS males to bondRNAi males perfumed with synthetic CH503 (effect size, Pearson’s r = 0.59) but was not fully rescued suggesting that other bond-related sensory cues are used for assessing rivals.

Tissue-specific expression of bond splice variants. Given the diverse functional roles of bond in pheromone production and male fertility, we investigated the genetic regulation of bond expression in various tissues from adult males and females. The gene is predicted to produce three transcripts, RB, RA and RC. Each transcript encodes an identical protein but differs in the first non-coding exon (Fig. 3a). RB is the primary transcript in the EB of CantonS and bondRNAi males, with expression levels nearly two orders of magnitude greater than the other transcripts (Fig. 3b,c). In contrast, the RA transcript is more highly expressed in other male reproductive organs (testes and accessory glands). In bondRNAi males, the PBac sequence was inserted within the intronic region of RB resulting in a loss of RB expression in the EB and other tissues (Fig. 3a). Notably, RA and RC expression could still be detected in the EB and throughout the fly (Fig. 3b). Thus, on the basis of the high relative levels of RB expression in the EB and the observation that RB expression alone is lost in the EB of bondRNAi males, we conclude that the product of the bond RB transcript is essential for CH503 production. In females, the RB bond transcript was evident in the reproductive organs, consistent with previous findings. A long non-coding RNA, CR44062, is predicted to overlap with the RB region but the transcript was not detected in wild-type or transgenic flies and is thus, unlikely to contribute to CH503 production (Supplementary Fig. 3).

Age-dependent increase in bond expression and CH503. To determine if bond expression is correlated with sexual maturity, we compared CH503 amounts and bond transcript levels from flies at different ages (Fig. 3c). Production of CH503 increases with age: recently-eclosed males produce only trace amounts of the pheromone in the EB while sexually mature males produce approximately 533.7 ± 137.3 ng (Fig. 3c). Analysis by quantitative PCR (qPCR) of EB extracts reveal a spike in RB transcript expression at 2 days old, concomitant with a rise in CH503 levels, and a subsequent decrease at 5 days old.

Bond expression in reproductive organs. Next, we examined the expression pattern of the bond transcript and protein in the male testes and EB using RNA in situ hybridization (ISH) and immunostaining with an antibody generated to the Bond protein. Both methods revealed expression in the EB wall epithelium (Fig. 4a). Antibody staining also showed labelling of the accessory glands and spermatid heads within the testes, consistent with previous observations (Supplementary Fig. 4). Positive
immunostaining was not observed in the EB of bond^{0367} mutants but was evident in bond^{e0367} males (Supplementary Fig. 4).

**Bond expression in the EB enables greater chemical diversity.** To determine whether the role of Bond in the synthesis of CH503 or other very long-chain fatty acyl pheromones is conserved, we examined expression of the bond transcript and protein in the reproductive tissues of different drosophilid species. Previous work has shown that males of *D. simulans* and *D. yakuba* produce CH503 while *D. ananassae*, *D. willistoni* and *D. erecta* do not (Fig. 4a). As predicted, expression of bond messenger RNA and protein is evident in the EB of *D. simulans* and *D. yakuba* (Fig. 4a). Interestingly, the EBs of two non-CH503-producing species, *D. erecta* and *D. ananassae*, also showed positive ISH staining although the signal was much weaker. Positive signal for bond by ISH labelling or immunostaining was not observed in *D. willistoni* (Fig. 4b).

We next asked whether bond expression in the EB of other species is encoded specifically by the RB transcript, as is the case for *D. melanogaster*. Alignment of the bond locus identified a putative homologous sequence for the non-coding exon of the RB transcript in each of the drosophilids (Supplementary Fig. 5). Semi-qPCR analysis using primers specific to the RB transcript revealed expression in the EB of *D. melanogaster*, *D. simulans*, *D. yakuba*, *D. erecta* and *D. ananassae* but not *D. willistoni*. Notably, RB transcript expression in the testes of all species is comparatively weaker or not detected (Fig. 4b; Supplementary Fig. 4). In contrast, the exon-coding region of bond or the protein product is robustly detected in the testes of all species tested, indicating regulation by the RA or RB transcript splice form (Fig. 4c; Supplementary Fig. 4). The bond exon signal from *D. simulans* testes is relatively weak; however, both ISH and immunostaining revealed robust expression of the bond transcript and protein (Fig. 4a; Supplementary Fig. 4). Taken together, these results show that bond expression is conserved across six drosophilid species in the reproductive organs used for spermatogenesis. However, expression in the EB wall epithelium occurs only in some species and appears to be under control of the bond RB splice variant.

**Discussion**

The VLCFA elongase Bond was previously shown to be important for spermatogenesis. In this study, we identify a second role for Bond in the production of the sex pheromone CH503.
CH503. We show also that the products of bond are essential for male fertility and modulating the effects of male rivalry on fertility. The gain of a novel transcription initiation site is correlated with the gain of bond expression in the EB, thus enabling Drosophila males to produce CH503 and other fatty acyl long-chain pheromones.

The Bond elongase belongs to the Elovl family of enzymes, which are conserved from yeast to mammals, and are involved in the elongation of long-chain fatty acids and VLCFA (longer than 22 carbon atoms). The VLCFA s are needed for the production of sphingolipids, an integral membrane component of many cell types including spermatocytes, myelin, sebaceous glands and hepatocytes. The substrates for many cell types including spermatocytes, myelin, sebaceous glands and hepatocytes are not known; however, the bond locus and site of PBac-element insertion predicts three transcripts: RA, RB and RC. Purple triangle: site of PBac insertion in bond flies; grey boxes: non-coding exons; and red boxes: coding exons. (b) Semi-quantitative PCR with reverse transcription reveals differential expression of the bond transcripts in adult tissues from males and females. Of the three transcripts, RB is most highly expressed in the EB of CantonS and bond flies and was not detected in bond flies. The rp49 gene was used as a loading control. (c) Quantitative real-time PCR and HPTLC analysis of EB extracts from CantonS males at 0, 2 and 5 days old. Each point represents mean ± s.e.m.; ANOVA with Kruskal–Wallis test, **P < 0.01, n = 3. For CH503 quantitation, bars represent mean ± s.e.m., three replicate extracts from 20 EBs each. ANOVA, analysis of variance.

Figure 3 | bond transcript expression in the ejaculatory bulb is age dependent and necessary for CH503 production (a) Genetic organization of the bond locus and site of PBac-element insertion predicts three transcripts: RA, RB and RC. Purple triangle: site of PBac insertion in bond flies; grey boxes: non-coding exons; and red boxes: coding exons. (b) Semi-quantitative PCR with reverse transcription reveals differential expression of the bond transcripts in adult tissues from males and females. Of the three transcripts, RB is most highly expressed in the EB of CantonS and bond flies and was not detected in bond flies. The rp49 gene was used as a loading control. (c) Quantitative real-time PCR and HPTLC analysis of EB extracts from CantonS males at 0, 2 and 5 days old. Each point represents mean ± s.e.m.; ANOVA with Kruskal–Wallis test, **P < 0.01, n = 3. For CH503 quantitation, bars represent mean ± s.e.m., three replicate extracts from 20 EBs each. ANOVA, analysis of variance.
species tested, both the bond transcript and protein are found in the EB. Unexpectedly, bond expression was also observed in non-CH503-producing species D. erecta and D. ananassae. It may be the case that bond contributes to the production of other long-chain fatty acid-based pheromones observed in the EB profiles (for example, the molecule with m/z 407 found in the EB profile of both species, Fig. 4a).

Several mechanisms have been proposed to underlie the evolution of pheromone biosynthesis enzymes. Most studies support the birth-and-death model of gene duplication, independent gene loss and pseudogenization. For example, in drosophilids, the desat1, desat2 and desatF clusters were formed from gene duplication with desat1 being the most ancient copy. More recently, desat2 was independently rendered

Figure 4 | Comparative analysis of bond expression in male reproductive organs. (a) First column: linear parsimony diagram depicting relations of the drosophilid species used in this study. CH503-producing species are highlighted in red. Second column: RNA in situ hybridization of bond transcript in the EB of adult males. Arrowheads indicate positive signal in the epithelium (in purple). Third column: arrowheads indicate positive anti-Bond immunostaining (in green) in the EB. Fourth column: UV-LDI mass spectra from the EB reveal that the production of CH503 is limited to D. simulans, D. melanogaster and D. yakuba. Other unidentified lipids are observed in the EB of D. ananassae and D. willistoni. Scale bar, 50 μM. (b) PCR amplification of the bond RB transcript from EB and testes tissue extracts. The rp49 gene was used as a loading control. (c) PCR amplification of the bond open-reading frame (ORF) from testes tissue extracts.
nonfunctional in the Canton S strain but not the Tai strain of *D. melanogaster*. Studies of desaturases in corn borer moths indicated a second mechanism involving the fusion of a retrotransposon long interspersed element and desaturase to give new functions. Finally, co-option has been suggested as a third mechanism whereby phenotypic features could diversify. Modification of *trans-* and/or *cis-*regulatory sequences is known to cause expression of a gene in a new tissue and use for a new function. On the basis of our observations that *D. willistoni* expresses bond in the testes but not EB, it is possible that bond first originated as an elongase involved in spermatogenesis. The gain of a novel transcription initiation site (*bonde*03675) allowed regulatory sequences is known to cause expression of a gene in a new tissue and use for a new function. The *bonde*03675 expression in the testes but not EB, it is possible that bond first originated as an elongase involved in spermatogenesis.

**Methods**

*Drosophila stocks and husbandry*. All flies were raised on standard fly food (autoclaved cornmeal, yeast, sucrose, agar, food) at a 12-h-light/dark cycle, unless stated otherwise. The day of eclosion is designated as day 0 for adult fly age. The following lines were used: isogenic Canton S line (9517), Berlin strain, PiggyBac (PBac) insertion line bond*03675* (18181), 97a06125 (189499), UAS-CaMPS (32126) and UAS-mCD8::GFP (5157) from Bloomington *Drosophila* Stock Center; NP0984-GAL4 (V104866, DGRG); and UAS-bondeVIAI (v30179, VDRC). All non-melanogaster species were obtained from the UCD *Drosophila* Species Stock Center.

**Transgenic lines.** The bond PBac insertion line bond*03675* was generated by crossing bond*03675* males to female flies bearing a ubiquitously expressed transposase (#283, BDSC). F1 males with mosaic eyes were recovered and backcrossed to bond*03675* 67. White-eyed flies were collected and backcrossed to bond*03675* 67 to generate the bond-Gal4 line, a 1.5-kb genomic fragment of the region upstream from the promoter of the bond RB transcript was synthesized (Genscript, USA) and inserted into a pChs-GAL4 plasmid using the restriction enzymes KpnI (5′) and Sall (3′). To generate the UAS-bonde line, the transgene region of bond including introns was cloned into a pUAST-attPlasmid with BglII (5′) and NotI (3′) (Genscript, USA). The plasmids were injected into *Drosophila* embryos.

**High-performance thin-layer chromatography.** EB extract was prepared by extracting dissected bulbs with hexane (Fisher Scientific, New Jersey, USA) under long-wave ultraviolet (66:33:1) and visualized with 0.02% primuline (w/v in 20% acetone; Sigma-Aldrich, USA). The plasmids were injected into *Drosophila* embryos.

**Ultraviolet-laser desorption ionization mass spectrometry.** For intact fly analysis, individual flies were attached to a custom-milled sample plate using double-sided tissue tape (7111, Louis Adhesive Tapes, Thailand). No matrix was used. For fly-assisted laser desorption/ionization, samples (or extract) were placed onto *Drosophila* wings washed with chloroform: methanol (1:1), v/v, before exposure to a xenon lamp. To prevent cross contamination, each tissue type was placed on a separate wing. More information about the application of fly-assisted laser desorption/ionization can be found in refs 13, 18, 46. Mass spectra were generated using a QSTAR Elite (AB Sciex, Concord, Ontario, Canada) mass spectrometer equipped with a modified oMALDI ion source (AB Sciex) and a N2 laser (337 nm) operated at a repetition rate of 40 Hz. Two mbar of N2 gas was used create the buffer gas environment for generation of ions. ([M + K]⁺ potassium-bearing compounds constitute the major ion species. Spectra were internally calibrated to chitin signals at *m/z* 242.04, 648.20 and 851.28. Mass accuracy was ∼20 ppm. All spectra were acquired in positive ion mode and processed using MS Analyst software (Analyst Q5 2.0, AB Sciex).

**Gas chromatography mass spectrometry.** Extracts were prepared by placing eight flies in hexane containing 10 µg/ml 1-hexacosane for 10 min at room temperature. The extract was evaporated with *N*₂ in a clean glass vial and stored at −20 °C until analysis.). Analysis was run in a 5% phenyl-methylpolysiloxane (DB-5, 30-m length, 0.32 µl, 0.25-µm-film thickness, Agilent) column and GCMS QP2010 system (Shimadzu) with an initial column temperature of 50 °C for 2 min and then increased to 300 °C at a rate of 15 °C min⁻¹ in splitless mode. Statistical analysis of GCMS results and fertility assays was performed using Prism, v. 6 (GraphPad, La Jolla, CA, USA).

**Quantitative analysis of mRNA transcripts.** The dorsal abdomen was dissected by separating the abdomen from the thorax and removing as much of the internal organs as possible without disrupting the oenocytes on the inner dorsal surface. The subcuticular tissues include the integument and seminal vesicles. The testes tissues include the testes and seminal vesicles. The accessory gland and EB tissues usually contains part of the ejaculatory duct. The female reproductive organ includes the ovaries and the uterus. Total RNA was prepared using TRIzol Reagent according to the manufacturer's instructions. A concentration between 100–300 ng µl⁻¹ was usually obtained. A 200 ng complementary DNA (cDNA) was prepared using SuperScript III Reverse Transcriptase (18080-044, Life Technologies, USA). Semi-qPCR was performed with 30 ng cDNA using Taq DNA Polymerase (11446176001, Roche, Switzerland) according to the manufacturer's instructions. The annealing temperature was 58 °C; 25 cycles were performed. See Supplementary Table 1 for primer sequences. For qPCR, the extracted RNA from the EB was treated with TURBO DNA-free Kit (AM1907, Life Technologies, USA) before reverse transcription reaction (as above). A amount of 5 ng of cDNA was used for qPCR, using KAPA SYBR FAST qPCR Kit (KK4604, KAPA Biosystems, USA) and Applied Biosystems 7900HT Fast Real-Time PCR system. The conditions used were as recommended for *Drosophila* by KAPA SYBR FAST and the PCR products were sequenced across the forward and reverse primers. The mean and s.e.m. calculated from normalized values. An analysis of variance comparing the normalized levels at each point was performed using Prism, v. 5 (GraphPad, La Jolla, CA, USA). See Supplementary Table 1 for primer sequences.

**RNA in situ hybridization.** To generate the RNA probe, primers with Sp6 (5′-GATTAGTGACATTACTAG-3′) or T7 (5′-TAATACGACTCACTATAGGG-3′) RNA polymerase promoter sequences tagged to the 5′-end were designed to amplify an approximate 500-bp region of the third exon and used at an annealing temperature of 58 °C. The PCR products were transcribed with either Sp6 or T7 RNA polymerase and DIG RNA labelling mix (11277073910, Roche, Germany). Primer sequences can be found in Supplementary Table 1. The tissues were dissected in phosphate buffered saline (PBS), fixed in PBS/formaldehyde (v/v, 2:1) for 20 min, washed with PBST (0.05% Tween 20 in PBS, pH 7.4) and treated with Proteinase K (50 µg ml⁻¹). A concentration between 100–300 ng µl⁻¹ of KAPA SYBR FAST qPCR Kit. Relative transcript levels were calculated as 2^ΔΔCt. ΔCt = Ctarget - Cinternal control.

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**Immunostaining.** Mouse monoclonal antibodies raised against the peptide sequence LQNQGVGKAY from the Bond protein were generated (Abmart, China). Tissues were dissected in PBS, fixed in 4% paraformaldehyde in PBS (v/v) for 30 min at room temperature, washed in PBS and incubated at room temperature for 5 h with the anti-Bond antibody (diluted 1:1,000 in PBS). For blocking solution containing 0.025% sodium azide and 5% NGS in PBS (0.1% Triton X-100 in PBS, pH 7.4) before incubating with anti-Digoxigenin-AP (11093274910, Roche, Germany) for 1 h. Following washes in PBS and 0.1% Tween 20 in AP buffer (0.1 M Tris (pH 9.5), 0.2 M NaCl and 50 mM MgCl2), the tissues were incubated with NBT-BCIP substrate (B1911, Sigma-Aldrich, St Louis, USA). A brief wash in PBS was used to stop the reaction. The tissues were mounted with 50% glycerol in PBS and imaging was performed with Leica MZ10 F modular stereo microscope.

**Female choice assay.** Six *bonde*03675 males were placed in a 1.8-ml glass vial (Wheaton, NJ, USA) containing evaporated hexane, 83 ng CH503, or 830 ng (S,Z)-CH503 (kind gifts of K. Mori, Toyo-Gosei Company, Japan; synthesis described in refs 45, 48). The flies were vortexed three times with 20-s rest intervals. Following a 1-h recovery period, two males (one of each treatment, differentiated by marking the wings) and one virgin female were placed in a 16 × 9-mm courtship chamber and their behaviour monitored for 30 min. Female choice is defined as the first male with which the female mated. Trials in which females did not mate
within 60 min were excluded. Preference index is represented as total trials for one choice (perfumed or non-perfumed) normalized to the total number of trials. Results were analysed using a one-tailed Fisher exact test (Vassar Stats: vassarstats.net), comparing experimental results to null condition (no preference, 50:50 distribution of choices). Effect size was calculated from the odds ratio 39.

**Fertility assay.** Males were isolated from pupal stage in test tubes containing 2 ml fly food. CantonS virgin females were housed groups of 10. At 5 days old, a single male and virgin female were placed in a test tube for mating. The mated females were kept in food vials in three replicate groups of five flies each. Fresh vials were provided every 2–3 days. The total number of adult progeny from all vials was counted.

**Rivalry assay.** CantonS males were isolated from pupal stage as above in test tubes containing 2 ml fly food. At 3 days old, three rival males were introduced. The wings of the rivals were marked for identification. To eliminate visual signals as a rivalry cue, the introduction of rivals and rival incubation was performed in a dark room with red light. After 2 days, the CantonS male was placed in a fresh test tube and allowed to acclimatize to light for an hour. A virgin CantonS female was introduced and the length of copulation scored. Fertility was scored as described for the fertility assay.

For all behavioural assays, post hoc power analysis using GPower 3 (ref. 50) indicated that the sample sizes were adequate to achieve a statistical power of at least 0.8 for an z-level of 0.05.

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Author contribution
W.C.N. and J.Y.Y. designed the experiments. W.C.N., J.S.R.C., K.J.T. and J.Y.Y. conducted the experiments and analysed the data. W.C.N., J.S.R.C., K.J.T. and J.Y.Y. wrote the manuscript.

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