Howard Hughes Medical Institute

Published as: *Nat Neurosci*. 2011 June ; 14(6): 757–762.

Hierarchical chemosensory regulation of male-male social interactions in *Drosophila*

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Abstract

Pheromones regulate male social behaviors in *Drosophila*, but the identities and behavioral role(s) of these chemosensory signals, and how they interact, are incompletely understood. Here we show that (*Z*)-7-tricosene (7-T), a male-enriched cuticular hydrocarbon (CH) previously shown to inhibit male-male courtship, is also essential for normal levels of aggression. The opposite influences of 7-T on aggression and courtship are independent, but both require the gustatory receptor Gr32a. Surprisingly, sensitivity to 7-T is required for the aggression-promoting effect of 11-*cis*-vaccenyl acetate (cVA), an olfactory pheromone, but 7-T sensitivity is independent of cVA. 7-T and cVA therefore regulate aggression in a hierarchical manner. Furthermore, the increased courtship caused by depletion of male CHs is suppressed by a mutation in the olfactory receptor *Or47b*. Thus, male social behaviors are controlled by gustatory pheromones that promote and suppress aggression and courtship, respectively, and whose influences are dominant to olfactory pheromones that enhance these behaviors.

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AUTHOR CONTRIBUTIONS

L.W. carried out the experiments and performed the data analysis. L.W. and D.J.A. together conceived the research and wrote the manuscript. X.H. synthesized CH molecules. J. M. generated and characterized *Or47b* mutant alleles. M. H. characterized the expression of *Gr32a-GAL4*. J-C.B., T.M., H.A. and J.D.L. contributed fly strains.

INTRODUCTION

Social interactions between male *Drosophila* involve relatively high levels of aggression, and low levels of courtship[1, 2]. How the normal balance between these two behaviors is achieved is poorly understood. Previous studies have implicated pheromones detected by both the gustatory and olfactory systems in regulating these social interactions. Males lacking all gustatory sensilla[3], specific gustatory receptors[4, 5], or their normal complement of CHs[6, 7], non-volatile pheromones that include ligands of gustatory receptors[8], exhibit elevated levels of male-male courtship. These data suggest that gustatory pheromones play a suppressive role in male-male courtship. Conversely, a genetic manipulation that eliminates CHs[6] has recently been suggested to reduce male-male aggression[9]. Whether the same or different CH molecules inversely regulate these two male social behaviors is not clear.

A single olfactory pheromone, cVA, has been implicated in both suppressing male-male courtship and enhancing male-male aggression. Mutants lacking *Or67d*[10], a receptor for cVA[10–12], have been reported to exhibit elevated male-male courtship, implying that cVA may ordinarily suppress this behavior. However, a subsequent study is unable to replicate this observation[3], and exogenously applied cVA does not reduce male-male courtship[13]. In contrast, cVA strongly promotes male-male aggression, in an *Or67d*-dependent manner[13]. Although detection of cVA by Or67d receptor is not essential for aggression between isolated pairs of male flies, it may play a role under conditions of high male population density[13].

Despite this recent progress, the nature of the chemosensory signals that control the normal balance between male-male aggression and courtship is incompletely understood. Furthermore, the extent to which gustatory and olfactory systems function independently vs. interdependently or hierarchically to regulate these behaviors has not been investigated. In the present study, we have investigated the interplay between the gustatory and olfactory pheromones in the regulation of male-male social behaviors (Supplementary Fig. 1).

RESULTS

Male CHs control the balance between male social behaviors

We first asked whether male CHs were required for male-male aggression. Previous studies show that the production of these non-volatile pheromones requires oenocytes, a group of cells located beneath the abdominal cuticle of male flies[6, 14]. Male oenocytes can be selectively ablated in adults using genetic tools[6] (see Methods). This manipulation eliminated most if not all male CHs[6], including the two most abundant male-enriched CH molecules[8, 15, 16] (7-T and (*Z*)-7-pentacosene, 7-P), without affecting the levels of cVA, an olfactory pheromone synthesized in the male ejaculatory bulb[17] (Supplementary Fig. 2a, b and Fig. 1a, orange vs. blue bars). Strikingly, pairs of oenocyte-eliminated (oe^-) males exhibited significantly reduced levels of male-male aggression, compared to pairs of control (oe^+) males (Supplementary Fig. 3a), in addition to higher levels of male-male courtship[6] (Supplementary Fig. 3b). However, aggression between such oe^- males was not completely eliminated, consistent with a recent report[9].

To distinguish whether eliminating male CHs influenced the social behaviors of oe$^-$ males in a fly-autonomous or non-autonomous manner, we paired a wild-type Canton-S "tester" male with either an oe$^+$ or an oe$^-$ "target" male. Canton-S testers performed significantly lower levels of male-male aggression towards oe$^-$ than towards oe$^+$ targets, as measured by the occurrence of lunges (Fig. 1b, orange vs. blue bars; also see Supplementary Fig. 4). Wing threat, a behavioral display exhibited during male-male aggressive encounters[1, 18, 19], was not affected by the elimination of male CHs (data not shown). Canton-S tester males also performed significantly higher levels of courtship towards oe$^-$ than towards oe$^+$ target males, as measured by the occurrence of unilateral wing extensions (Fig. 1c, orange vs. blue bars) and circling episodes[2] (data not shown). To prove that the alteration of tester male behaviors was due to the lack of CHs on oe$^-$ target males, and not to some other, unknown effect of this genetic manipulation, we restored normal levels of male CHs to oe$^-$ males by passive transfer from control males[7] (Supplementary Fig. 2c and Fig. 1a, green bars; see Methods). This manipulation rescued both the decreased male-male aggression and increased male-male courtship (Fig. 1b, c, green bars; also see Supplementary Fig. 4) exhibited by the testers. These data indicate that male CHs not only inhibit male-male courtship, but positively regulate aggression as well.

The reciprocal influences of male CHs could reflect independent and opposite effects on male-male aggression vs. male-male courtship, or a primary effect exclusively on one behavior, which then indirectly inhibits the performance of the other. cVA can strongly enhance aggression without causing a concomitant decrease in male-male courtship[13], implying that aggression does not behaviorally inhibit male-male courtship. To ask whether, conversely, enhancing male-male courtship indirectly decreased aggression, oe$^+$ target males were perfumed with synthetic (*Z,Z)*-7,11-heptacosadiene (7,11-HD), a typical female-specific CH molecule[6, 8] (Supplementary Fig. 5 and Fig. 1d). This treatment elevated courtship by wild-type tester males towards the targets, without reducing their level of aggression (Fig. 1e, f). Thus elevated male-male courtship does not behaviorally inhibit aggression. The reciprocal effects of CHs on male-male aggression vs. male-male courtship therefore reflect parallel, direct influences of such pheromones on these two social behaviors.

7-T reciprocally regulates male aggression and courtship

Male CHs are comprised of multiple classes of compounds[8, 16]; among these, 7-T has been shown to suppress courtship[6, 20]. The requirement of CHs for normal levels of male-male aggression may therefore reflect the influence of a different male-enriched CH(s). Alternatively, 7-T might both promote aggression and suppress male-male courtship. To distinguish between these alternatives, we chemically synthesized 7-T and 7-P, the two most abundant male-enriched CHs[8], and asked whether perfuming oe$^-$ targets with either of them was sufficient to restore the normal balance of social behaviors by male testers (Supplementary Fig. 6 and Fig. 2a; see Methods). Remarkably, synthetic 7-T was sufficient both to restore normal levels of aggression, as well as to suppress courtship (Fig. 2b, c, green bars), by wild-type tester males. In contrast, synthetic 7-P exhibited no behavioral effect in this assay (Fig. 2b, c, purple bars). These data indicate that a single CH species can

exert opposite-direction influences on aggression and male-male courtship, in the absence of other CH molecules synthesized by oenocytes.

We next investigated whether these distinct influences of 7-T might be exerted at different concentrations of the pheromone. We generated oe^- target males carrying different amounts of 7-T, ranging from ~20% to ~2 fold of the amount of 7-T present on oe^+ males (Supplementary Fig. 7 and Fig. 2d; see Methods). These flies were then paired with wild-type tester males. Surprisingly, as little as ~30% of the wild type amount of 7-T applied to oe^- males could produce a significant increase in aggression and exhibited a trend to inhibit courtship (Fig. 2e, f). Further increasing the level of 7-T, to ~2-fold of the normal level, did not cause additional changes in either social behavior. These data indicate that 7-T oppositely influences aggression and inter-male courtship over a similar concentration range.

Gr32a mediates the behavioral effects of 7-T

Next, we investigated the chemosensory receptor(s) that mediates the behavioral effects of 7-T. Previous work has shown that 7-T can activate bitter-sensing GRNs[20], which express multiple gustatory receptors[21–23], but no specific receptor for 7-T has yet been identified. The fact that Gr32a is expressed in bitter-sensing GRNs[21–25], and that *Gr32a*$^{-/-}$ mutant males exhibit increased courtship towards decapitated males[4], suggests Gr32a as a candidate receptor mediating the behavioral effects of 7-T.

We first asked whether *Gr32a* was required for normal levels of aggression. When paired with oe^+ target males, *Gr32a*$^{-/-}$ mutant tester males[4] showed a diminished aggression level compared to *Gr32a*$^{+/-}$ heterozygous control males (Fig. 3a, *Gr32a*$^{+/-}$ vs. *Gr32a*$^{-/-}$; also see Supplementary Fig. 8). This *Gr32a* mutant phenotype was reverted by expression of a *Gr32a* genomic rescue construct[4] (Fig. 3a, *Gr32a*$^{-/-}$ *Rescue*; also see Supplementary Fig. 8). In contrast, the *Gr32a*$^{-/-}$ mutation did not affect the level of courtship towards oe^+ target males (Fig. 3b), although it did impair courtship towards oe^- target males perfumed with 7-T (see below). Although a previous study reports that *Gr32a*$^{-/-}$ mutant males show increased male-male courtship towards decapitated target males[4], a result that we independently replicated (Supplementary Fig. 9), such decapitated male targets may fail to provide additional signals, such as behavioral feedback[3], that intact males normally provide to suppress male-male courtship. It has also been reported that *Gr32a*$^{-/-}$ mutant males exhibit increased bilateral wing extension behaviors towards females[26], but we did not observe such behavior towards oe^+ male targets.

We then asked whether *Gr32a* is required for the effects of 7-T to promote aggression and inhibit male-male courtship. Indeed, *Gr32a*$^{-/-}$ mutant tester males failed to show increased aggression and decreased courtship towards oe^- targets perfumed with 7-T (Fig. 3c, d, *Gr32a*$^{-/-}$, orange vs. green bars). In contrast, control *Gr32a*$^{+/-}$ tester males, like wild-type males, exhibited increased aggression and decreased courtship towards such target males (Fig. 3c, d, *Gr32a*$^{+/-}$, green bars), at levels comparable to those displayed towards oe^+ targets (Fig. 3c, d, *Gr32a*$^{+/-}$, orange bars vs. dashed lines). The phenotype of *Gr32a*$^{-/-}$ mutants could be reverted by *Gr32a* genomic rescue (Fig. 3c, d, *Gr32a*$^{-/-}$ *Rescue*, orange vs. green bars). These data indicate that Gr32a is required for the inverse effects of 7-T on

aggression and male-male courtship (Supplementary Fig. 1, green), suggesting that *Gr32a* may encode a 7-T receptor. Whether Gr32a is required for the electrophysiological response to 7-T by GRNs[20] is an interesting question that remains to be investigated.

Notably, as fly GRNs often co-express >1 gustatory receptor[21–23], and as detection of bitter compounds in flies may require multiple receptors[25, 27], our data do not exclude the possibility that other gustatory receptors besides Gr32a are involved in the response to 7-T. Moreover, the fact that Gr32a is required for the suppression of male-male courtship by 7-T, but not by the full complement of male CHs present on oe^+ target males (Fig. 3b), likely indicates the existence of additional, functionally redundant CH species (e.g., CH503[28]), and receptor(s) other than Gr32a[5], that are involved in the suppression of male-male courtship (Supplementary Fig. 1, grey).

To test whether $Gr32a^+$ GRNs directly mediate both of the behavioral effects of 7-T, or simply play an indirect, permissive role, we asked whether artificial activation of these GRNs would be sufficient to restore aggression and suppress courtship towards oe^- target males. To do this, we ectopically expressed TRPV1, a mammalian capsaicin receptor[29] previously used to activate *Drosophila* GRNs[24], in $Gr32a^+$ GRNs. We paired *Gr32a-GAL4/UAS-TRPV1* tester males with oe^- target males, and asked whether applying capsaicin to these target males restored the normal balance of social behaviors exhibited by the TRPV1-expressing testers. Indeed, the presence of capsaicin on oe^- targets elicited aggression and suppressed courtship towards these targets by the *Gr32a-GAL4/UAS-TRPV1* testers (Supplementary Fig. 10), although the magnitude of this behavioral rescue was much lower than that obtained using 7-T. Control tester lines carrying either *Gr32a-GAL4* or *UAS-TRPV1* did not respond to capsaicin (Supplementary Fig. 10). Thus, artificial activation of $Gr32a^+$ GRNs in tester males partially mimicked the behavioral effects of 7-T. The incomplete penetrance of the capsaicin/TRPV1 manipulation likely reflects the expression of the *Gr32a-GAL4* driver[21] in only a subset of all $Gr32a^+$ GRNs[23] (M. H. and Kristin Scott, personal communications). However a requirement for activation of additional, $Gr32a^-$ GRNs to mimic a full behavioral response to 7-T, could also explain the difference. Whatever the case, the data suggest that 7-T is likely to act directly on $Gr32a^+$ GRNs to exert its opposing effects on aggression and male-male courtship (Supplementary Fig. 1, green).

7-T and cVA hierarchically regulate male aggression

The observation that 7-T plays a key role in aggression raised the question of how it interacted with cVA, an olfactory pheromone that regulates the intensity of male-male aggression[13]. Exogenous, synthetic cVA did not promote aggression in $Gr32a^{-/-}$ males, although it did so in $Gr32a^{+/-}$ control flies and in $Gr32a^{-/-}$ genomic rescue flies (Fig. 3e). The inability of synthetic cVA to promote aggression in $Gr32a^{-/-}$ mutant flies was particularly striking in light of the fact that these mutants still showed residual (yet greatly reduced) levels of aggression (Fig. 3a). Therefore, the failure of synthetic cVA to promote aggression of $Gr32a^{-/-}$ mutant flies cannot be simply ascribed to the absence of this behavior *per se*. Rather, the results imply that Gr32a-mediated signaling is required for (i.e., gates) the aggression-promoting effect of cVA. In contrast, the male-female courtship-

suppressing effect of cVA[10, 30] was undiminished in *Gr32a*$^{-/-}$ mutant males (data not shown). Thus the inability of cVA to promote aggression in *Gr32a*$^{-/-}$ mutants does not simply reflect a general insensitivity to cVA caused by *Gr32a* mutation.

We then asked whether, conversely, cVA sensitivity was required for the ability of 7-T to restore aggression towards oe^{-} targets. *Or67d*$^{GAL4/GAL4}$ mutant males are anosmic to cVA[10] and are insensitive to its aggression-promoting effect[13]. We paired them with oe^{-} targets or oe^{-} males perfumed with synthetic 7-T. *Or67d*$^{GAL4/GAL4}$ mutant males and the control *Or67d*$^{+/+}$ males showed comparable aggressive responses to 7-T (Fig. 3f). Similarly, ectopic expression of Kir2.1 in Or67d^{+} ORNs, which has been shown to eliminate the aggression-promoting effect of cVA[13], did not interfere with the aggressive responses to 7-T (Supplementary Fig. 11). Thus, sensitivity to cVA is not required for the ability of 7-T to restore normal levels of aggression towards oe^{-} males. This finding confirms and extends our previous observation that sensitivity to cVA is not required for normal levels of aggression between pairs of wild-type male flies[13]. Taken together, these data indicate that the aggression-promoting effect of cVA is dependent upon signaling through 7-T/Gr32a, but not vice versa, suggesting a hierarchical interaction between the gustatory and olfactory systems in regulating aggression (Supplementary Fig. 1, green and blue).

Or47b mutations suppress courtship toward oe^{-} males

The foregoing observations raised the question of whether hierarchical interactions between the gustatory and olfactory systems might also regulate male-male courtship. Specifically, we investigated whether the elevated levels of male-male courtship caused by genetic depletion of male CHs might require detection of olfactory pheromones. Previous studies have identified two olfactory receptors, Or47b and Or88a, that respond to odors present on both males and females[12]. Or47b^{+} ORNs express Fru^{M} and project to a sexually dimorphic glomerulus, VA1lm (also known as VA1v)[31, 32], suggesting a potential role in regulating social behaviors. Consistent with this idea, disruption of GABAergic signaling in Or47b^{+} ORNs has suggested a possible role for Or47b^{+} ORNs in the location of females by males[33]. We therefore investigated a possible role for Or47b in the elevated courtship exhibited towards oe^{-} target males.

We examined two independent *Or47b* null alleles, *Or47b*$^{2/2}$ and *Or47b*$^{3/3}$, generated by homologous recombination (see Methods) (Fig. 4a, b). *In situ* hybridization confirmed the elimination of *Or47b* mRNA in the third antennal segment, where it is normally expressed (Fig. 4c). The projections of Or47b^{+} ORNs to the VA1lm/VA1v glomerulus[31, 32] were unaffected by the mutation (Fig. 4d), indicating that lack of *Or47b* does not perturb proper targeting of these ORNs. Elimination of *Or47b* in tester males suppressed the elevated courtship exhibited towards oe^{-} target males (Fig. 4e), but did not affect the level of aggression (Fig. 4f). To determine whether this phenotype reflected a general deficit in courtship behavior, we also tested these mutants in male-female interactions. Both *Or47b*$^{2/2}$ and *Or47b*$^{3/3}$ mutant males exhibited normal latencies to copulate with virgin females, compared to *Or47b*$^{+/+}$ controls (Fig 4g). The frequency of unilateral wing extensions towards females also did not show a statistically significant difference among genotypes, although there was a trend, if anything, to a slightly higher level in the mutants (Fig. 4h).

These data reveal that the increased levels of male-male courtship caused by genetic depletion of male CHs can be suppressed by a mutation in *Or47b*. This implies the existence of one or more male courtship-promoting cues detected by this receptor, whose influence is normally subordinate to the courtship-suppressing effects of male CHs[6, 7] (Supplementary Fig. 1, green and red). It is also possible that the presence of male CHs suppresses the synthesis/release of pheromone(s) detected by Or47b. However this is an unlikely explanation given that odors from wild type males can activate Or47b[12]. The normal behavioral role of Or47b is not clear. Or47b and its unknown ligand may function in male-male behavior, e.g. by promoting social interactions that facilitate the detection of short-range chemosensory cues, such as 7-T. Effects of the *Or47b* mutation on male-female courtship have not yet been detected (J. D. L. and Leslie Vosshall, personal communications), but this receptor could play a role that is redundant with that of other olfactory (or non-chemosensory) cues (Fig. 4g, h).

DISCUSSION

The interplay between different chemosensory systems in the regulation of specific social behaviors is poorly understood. Here we provide evidence that non-volatile pheromones detected by the gustatory system dominantly control behavioral responses to olfactory cues that promote male-male social interactions. On the one hand, a male CH (specifically 7-T) is essential for the aggression-promoting influence of cVA; on the other hand, 7-T and other gustatory pheromones inhibit a courtship-promoting signaling pathway dependent upon Or47b.

The epistatic influences of male CHs on the behavioral effects of olfactory pheromones may be an indirect consequence of behavioral state changes, or may involve more direct sensory gating mechanisms (Supplementary Fig. 1, solid vs. dashed arrows). One way that male CHs could indirectly regulate the influence of olfactory pheromones is through a principal role in sex discrimination[6] (Supplementary Fig. 1, dashed arrows). According to this view, courtship and aggression reflect behavioral states that are automatically engaged as a consequence of recognizing the opponent fly as female vs. male, respectively. Consistent with this idea, masculinization of CH profiles can alter sex-specific patterns of male-female social interactions[9]. However, in the case of male-male social interactions, while oenocyte ablation increases male-male courtship[6] it does not fully eliminate male-male aggression (Fig. 1b, c). Since male aggression towards normal females almost never occurs[9], this residual aggression implies that male testers still recognize oenocyte-ablated targets as male. Therefore, instances of male-male courtship exhibited towards CH-depleted targets do not necessarily imply sex mis-identification (unless such identification must be made repeatedly upon each social encounter). The functional significance of courtship displays in male-male social encounters in *Drosophila* remains to be understood. In some arthropod species, male-male mating plays a role in establishing dominance[34], as it does in some human populations[35].

The foregoing considerations suggest that the effects of CH-depletion on male-male social interactions, and sensitivity to olfactory pheromones, may not be an indirect consequence of behavioral state changes caused by impaired sex discrimination. In that case, these gustatory

pheromones may regulate olfactory influences on these social behaviors via a more direct gating mechanism (Supplementary Fig. 1, solid green and black arrows). The circuit-level mechanisms by which such gating occurs will be an interesting topic for future investigation.

The chemosensory "logic" of male social interactions revealed here has some intriguing parallels in mice[36]. A mutation in *TrpC2*, which impairs the detection of pheromones by the vomeronasal organ (VNO[37]), results in both decreased male-male aggression and increased male-male courtship[38, 39]. By contrast, mutations affecting the main olfactory epithelium (MOE) reduce male-male aggression without increasing inter-male courtship[40, 41]. These phenotypes are similar to those caused by manipulating CH and cVA signaling, respectively, in *Drosophila*. This similarity suggests a division-of-labor between these two insect pheromonal systems that may be analogous to that between the accessory and main olfactory systems in vertebrates[42]. Major urinary proteins (MUPs) have recently been shown to function as murine aggression pheromones, and are detected by a subset of VNO neurons[43]. However their molecular receptor(s) remain to be identified. The identification and genetic manipulation of these and other pheromone-receptor pairs regulating aggression and courtship in mice should further clarify whether the hierarchical interactions revealed here represent a conserved "logic" for the chemosensory regulation of social behaviors.

METHODS

Fly stocks

Fly stocks were raised in vials containing standard fly medium made of yeast, corn and agar. The stocks were maintained in fly incubators at 25 °C and 60% humidity on a 12h:12h light-dark circle. In most cases, flies for behavioral assays were collected within 8 h after eclosion and were raised individually ("tester" males), or at 30 males/vial ("target" males) for 5–7 d before behavioral assays.

Canton-S flies were obtained from M. Heisenberg. *Gr32a*$^{-/-}$, the genomic rescue strains, and the *Gr32a-GAL4* strains[4] were from T. M. and H. A.. The fly strains for the ablation of oenocytes were from J-C. B. and J. D. L.. The *Or47b* mutant alleles were generated and characterized by J. M. at L. Vosshall's laboratory, and were backcrossed into the Canton-S background by J-C. B. and J. D. L..

Genetic elimination of male CHs

Ablation of male oenocytes[6] was achieved by crossing male "*+; PromE(800)-GAL4, tub-GAL80*^{TS}*; +*" flies to female "*+; UAS-StingerII, UAS-hid/CyO; +*" or female "*+; UAS-StingerII; +*" at 18 °C to generate oe^{-} or oe^{+} male progeny, respectively. Adult male progeny were collected within 8 h after eclosion and kept at 25 °C for 1 d. Subsequently, both oe^{+} and oe^{-} males were maintained at 30 °C during the daytime and at 25 °C during the nighttime for 3 more d. The flies were then maintained at 25 °C for 1–2 d before use. The expression of *UAS-hid*[44] in oenocytes specifically in the adult stage (under the control of *tub-GAL80*^{TS})[45] eliminates most if not all male CHs.

Behavioral assays

Unless otherwise indicated, the behavioral assays were performed using mixed fly pairs consisting of one single-housed "tester" male and one group-housed "target" male. As previously shown, group housing suppresses both aggression and courtship by male flies[2, 46]. Using a single-housed tester and a group-housed target male forces aggression and courtship to be conducted predominantly by the tester males. This uni-directional bias in the initiation of aggressive or courtship behaviors by the tester towards the target male facilitated analysis of the effects of experimental manipulations on the tester vs. the target.

To measure social interactions, one tester male and one target male were introduced into the behavior chamber (see below) by gentle aspiration. Their behavioral interactions were videotaped for 20 min and analyzed manually or by using custom CADABRA software[2]. To distinguish testers and targets, a blue dot was painted on the thorax of target males under CO_2 anesthesia, 1–2 d before behavioral assays were performed.

The design of the behavior arena was adapted from previous reports[2, 47]. Briefly, a rectangular chamber (4 cm × 5 cm × 12 cm) was placed on top of an acrylic base. In the center of the base, a 1 cm × 1 cm × 0.5 cm hole was filled with apple juice-agar-sucrose medium, surrounded by a 0.5 cm wide border containing 1% agar medium. The behavior arena was illuminated by a ring-shaped fluorescent lamp. Videotaping was performed using a commercial camcorder (Sony DCR-HC38) placed on top of the arena.

For experiments involving synthetic cVA, the experimental design was as previously reported[13]. Briefly, before and during behavioral assays, a small piece of filter paper containing 5 μl solvent (acetone) or 5 μl solvent containing 500 μg cVA was placed at one corner of the behavior arena. Male flies of the indicated genotypes were housed at 10 flies/vial for 5–7 d before behavioral assays. A pair of male flies of identical genotype and age was introduced into the behavior arena and their behavioral interactions were recorded for 20 min and analyzed by using custom CADABRA software.

For courtship assays between males and females, virgin Canton-S females were group-housed (10 flies/vial) for 5–7 d before behavioral assays. One tester male fly and one virgin Canton-S female were introduced into the behavior arena and their behavioral interactions were recorded for 10 min. The latency to copulate and the occurrence of one wing extensions were scored if applicable.

Chemical synthesis of CH molecules

(*Z*)-7-pentacosene (7-P) was synthesized as follows. *n*-BuLi (2.5 M in hexanes, 8.86 ml, 22.2 mmol) was added drop-wise to a solution of 1-octyne (2.22 g, 20.2 mmol) in THF (100 ml) at 78 °C and the mixture was stirred at 78 °C for 30 min and at 0 °C for 30 min. The resulting solution was treated with a solution of 1-bromoheptadecane (5.10 g, 16.0 mmol) in THF (10 ml), tetrabutylammonium iodide (0.74 g, 2.02 mmol), and refluxed for 15 h. The reaction mixture was quenched with saturated aqueous NH_4Cl and extracted with ether. The combined ether extracts were washed with brine, dried ($MgSO_4$), and concentrated under vacuum. The residue was chromoatographed (hexanes) to give 7-pentacosyne as a colorless viscous oil (2.93 g, 42 %).

To a solution of 7-pentacosyne (0.920 g, 2.64 mmol) in hexanes (35 ml) was added quinoline (0.853 g, 6.60 mmol) and Lindlar catalyst (0.562 g). The resulting suspension was vigorously stirred under a hydrogen balloon for 2 h. The catalyst was filtered off through a pad of Celite. The solvent was evaporated and the residue was purified by flash chromatography (hexanes) to afford (*Z*)-7-pentacosene as a colorless oil (0.823 g, 89%) with greater than 98% purity by gas chromatography.

(*Z*)-7-tricosene (7-T, greater than 98% purity by gas chromatography) was synthesized from 1-octyne and 1-bromopentadecane employing a procedure analogous to that used to synthesize (*Z*)-7-pentacosene.

(*Z*,*Z*)-7,11-heptacosadiene (7,11-HD) was prepared employing a modification of published procedures[48, 49]. A solution of methylmagnesium bromide (3.0 M in ether, 2.11 ml, 6.34 mmol) was added drop-wise to a stirring suspension of (dppp)$NiCl_2$ (1.56 g, 2.88 mmol) in benzene (40 ml) and the mixture was refluxed for 15 min. A solution of hexylmagnesium bromide (2.0 M in ether, 36.0 ml, 72.0 mmol) was added and most of the ether was removed by distillation under N_2. Benzene (100 mL) and dihydropyran (9.09 g, 108 mmoL) were added and mixture was refluxed for 16 h. The reaction mixture was quenched with saturated aqueous NH_4Cl and extracted with ether. The combined ether extracts were washed with brine, dried ($MgSO_4$), and concentrated under vacuum. The residue was twice chromatographed (hexanes/EtOAc = 20/1 to 10/1), the second time on silica gel embedded with $AgNO_3$ (10% w/w) to give (*Z*)-4-undecan-1-ol as a colorless viscous oil (0.675 g, 6 %).

A mixture of (*Z*)-4-undecan-1-ol (0.635 g, 3.73 mmol) and PCC (1.21 g, 5.60 mmol) was stirred in CH_2Cl_2 (10 ml) for 1 h. The suspension was diluted with ether, filtered through a pad of Celite, and concentrated under vacuum. The residue was chromoatographed (hexanes/EtOAc=10/1) to give (*Z*)-4-undecanal as a colorless oil (0.338 g, 54 %).

n-BuLi (2.5 M in hexanes, 0.63 ml, 1.57 mmol) was added drop-wise to a solution of hexadecyltriphenylphosphonium bromide (0.975 g, 1.72 mmol) in THF (30 ml) at −30 °C. The reaction mixture was allowed to warm to room temperature for 20 min, then cooled to −30 °C, HMPA (5 ml) was added, and cooled to −60 °C. A solution of (*Z*)-4-undecanal (0.240 g, 1.43 mmol) in THF (10 ml) was added drop-wise and the mixture was allowed to warm to room temperature. The reaction mixture was quenched with water and extracted with ether. The combined ether extracts were washed with brine, dried ($MgSO_4$), and concentrated under vacuum. The residue was chromoatographed (hexanes) to give (*Z*, *Z*)-7,11-heptacosadiene as a colorless oil (0.361 g, 67%) with greater than 98% purity by gas chromatography.

Quantification of male CHs

The quantification method was adapted from earlier reports[6, 14]. Briefly, individual male flies were CO_2 anesthetized and washed for 5 min in 25 μl of iso-octane containing 20 ng/μl of octadecane as an internal standard. The iso-octane extracts were analyzed by gas chromatography. 1 μl of each male CH extract was injected into a Hewlett-Packard 5890 II gas chromatograph coupled with a HP 5972 mass selective detector system. The injector was held at 300 °C and operated in splitless mode for 0.75 min after injection. A 30 m ×

0.25 mm ID × 0.25 μm film thickness RTX-5MS column from Restek Corporation (Bellafonte, Pennsylvania, US) was operated with a flow of 0.9 ml/min helium corresponding to a linear velocity of 34.4 cm/sec. The oven temperature began at 55 °C for 1.5 min and was ramped at 40 °C/min to 135 °C and then at 25 °C/min to 235 °C and then at 3 °C/min to 275 °C where it was held for 1 min. Electron impact spectra (70 eV electron energy) were recorded from 50 to 550 m/z at a rate of 1.5 scans per sec. HP Chemstation G1701 BA version B.01.00 software was used to calculate the retention time, the total peak area, and the identity of each compound.

Perfuming of male flies with CH molecules

The procedure for perfuming live oe^- males with male CHs was based on a passive transfer protocol adapted from a previous report[7]. Briefly, ten 5–6 d old oe^- male flies were mixed with 100 oe^+ males in small vials (~10 cm^3). The vials were placed upside-down in a 25 °C incubator for 1 d before behavioral assays or gas chromatography. Such a protocol ensured that a wild type-equivalent amount of male CH molecules was transferred to individual oe^- males. For behavioral assays, these oe^- males were marked by a blue dot on the thorax, which could be used to sort them out from oe^+ males without anesthesia. For gas chromatography, these males were instead marked by cutting off one wing before mixing.

The procedure for perfuming male flies with synthetic CH molecules was also adapted from a previous report[6]. Briefly, the compound of interest (2.5 μl for 7-T, 1 μl for 7-P and 1.5 μl for 7,11-HD, or no compound for control) was applied directly onto a small piece of filter paper in a 5 ml glass vial. Groups of 5–8 males were introduced into the vial by gentle aspiration, and vortexed twice at medium speed, each for 20 sec. The male flies were then transferred to fresh vials containing fly food. The vials were placed upside-down in the 25 °C incubator for 24 h before behavioral assays or gas chromatography. Such a protocol ensured that a wild type-equivalent amount of 7-T, 7-P or 7,11-HD was transferred to individual males.

To perfume different amounts of synthetic 7-T, an identical procedure was followed, except that the oe^- males carrying synthetic 7-T were allowed to recover in vials for 6 h, 24 h, 72 h and 96 h before behavioral assays or gas chromatography, resulting in the oe^- males carrying progressively smaller amounts of 7-T as a function of recovery time. Target males flies were used at a comparable age for behavioral assays or gas chromatography, independent of their post-transfer recovery times.

For experiments involving TRPV1, capsaicin (Sigma M2028) was dissolved in ethanol at 400mM, and subsequently diluted at 1:25 in acetone (adapted from ref. 24). 0.5 μl of this solution was carefully pipetted onto the abdomen of individual male flies under CO_2 anesthesia. For control males, the same procedure was applied, except that no capsaicin was added in the ethanol:acetone solution. Flies were transferred back to vials and were allowed to recover for ~12 h before behavioral experiments.

Generation of *Or47b* mutant alleles

Mutants for *Or47b* were generated by homologous recombination, using the "ends-out" technique[50], which replaces the exons of interest with a selectable marker, in this case the eye color pigmentation gene, *white*. Regions 5′ and 3′ of the gene were amplified by PCR from genomic DNA as follows:

5′ ARM 5.218 kb: Or47b.up-for and Or47b.up-rev

3′ ARM 3.369 kb: Or47b.dn-for and Or47b.dn-rev

Or47b.up-for	TCGCTTTTCGGCTTGTCT
Or47b.up-rev	TTGCGATGGATGGATAGG
Or47b.dn-for	CACCCACTCGCAAATGAA
Or47b.dn-rev	CATTTTCACCGCAACCTG

Fragments were subcloned into the CM105 (S. Chen and G. Struhl) vector which contains two polylinkers flanking the mini-*white* gene with a unique I-SceI site 5′ of the white gene, flanked by FRT sites and containing conventional P element repeats. The 5′ arm was cloned into the AvrII site and the 3′ arm was cloned into the NotI site. The construct was designed to delete sequences containing the first two exons and 1kb of DNA upstream of the translation start site. Virgin female flies carrying one targeting construct were crossed to *w118, 70FLP, 70I-SceI*, Sco/CyO and 3-day-old progeny were heat shocked at 38oC for 60 min. Homozygous transgenic lines were created by standard techniques. To check that the targeted homologous recombination took place, PCR primers Or47b.2-for and Or47b.2-rev were used to amplify sequence containing the first 2 exons of the *Or47b* gene, which were deleted in the null mutant. Primers Or47b.3-for and Or47b.3-rev were used to amplify sequence containing the last 4 exons of the *Or47b* gene, which is intact in the *Or47b* null mutants.

Or47b.2-for	CATGTGCAATGTGATGACCA
Or47b.2-rev	CGATGCAAAGCAACTTGAGA
Or47b.3-for	TCAAGTTGCTTTGCATCGAG
Or47b.3-rev	ATGCAAATGGCCAGAAAAAG

Statistical analysis

Most of the behavioral data were non-parametrically distributed. Mann–Whitney U tests (for pair-wise comparisons) and Kruskal-Wallis analysis of variance (ANOVA) (for comparisons among >2 groups) were applied. Significant difference among groups detected by Kruskal-Wallis ANOVA was analyzed using Dunn's *post hoc* test (with corrections for multiple comparisons) to identify groups with statistically significant differences. Two-way ANOVA was applied for comparisons among cumulative copulation latency curves.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Anderson lab members for helpful discussions, G. Mancuso for administrative assistance, G. Mosconi for lab management, and K. Scott and L. Vosshall for critical comments on the manuscript. Assistance from Dr. Nathan F. Dalleska and use of GC-MS instrumentation in the Environmental Analysis Center at the California Institute of Technology is gratefully acknowledged. We thank L. Vosshall for generously providing the *Or47b* mutant alleles generated by J.M. in her laboratory. D.J.A. is an investigator of the Howard Hughes Medical Institute.

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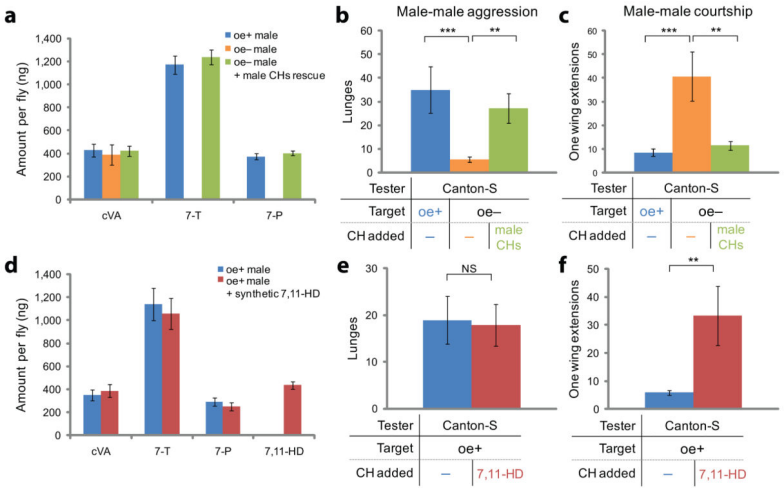

Figure 1. Male CHs are important for the normal balance of male-male social behaviors

(a) Quantification of cVA and major CH molecules from oe$^+$ (blue), oe$^-$ (orange) or oe$^-$ males perfumed with male CHs (green) (n=10). (b, c) Quantification of aggression (b) and courtship (c) performed by Canton-S tester males towards target males of the indicated genotypes and CH perfuming treatments (n=20). (d) Quantification of cVA and major CH molecules from oe$^+$ (blue) or oe$^+$ males perfumed with synthetic 7,11-HD (red) (n=9). (e, f) Quantification of aggression (e) and courtship (f) performed by Canton-S tester males towards target males of the indicated genotypes and CH perfuming treatments (n=16). Error bars are s.e.m. in this and all subsequent figures. NS: p>0.05, *p<0.05, **p<0.01 and ***p<0.001.

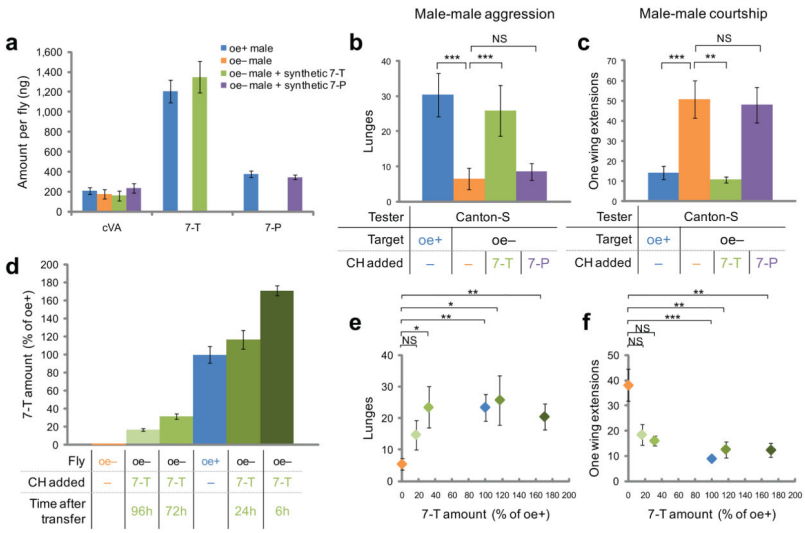

Figure 2. 7-T reciprocally regulates both male-male aggression and courtship

(a) Quantification of cVA and major CH molecules from oe^{+} (blue), oe^{-} (orange) or oe^{-} males perfumed with synthetic 7-T (green) or 7-P (purple) (n=11–12). (b, c) Quantification of aggression (b) and courtship (c) performed by Canton-S tester males towards target males of the indicated genotypes and CH perfuming treatments (n=20). (d) Relative levels of 7-T carried by oe^{-} males incubated after 7-T transfer for various periods of time (green), shown as a percentage of wild-type levels of 7-T (blue) (n=9–11). Absolute quantification is shown in Supplementary Fig. 6. Green shading represents relative amount of synthetic 7-T carried by oe^{-} males (darker = higher). (e, f) Quantification of aggression (e) and courtship (f) performed by Canton-S tester males towards oe^{-} targets (orange), oe^{+} targets (blue), or oe^{-} target males carrying different amounts of synthetic 7-T (green) (n=18). NS: p>0.05, *p<0.05, **p<0.01 and ***p<0.001.

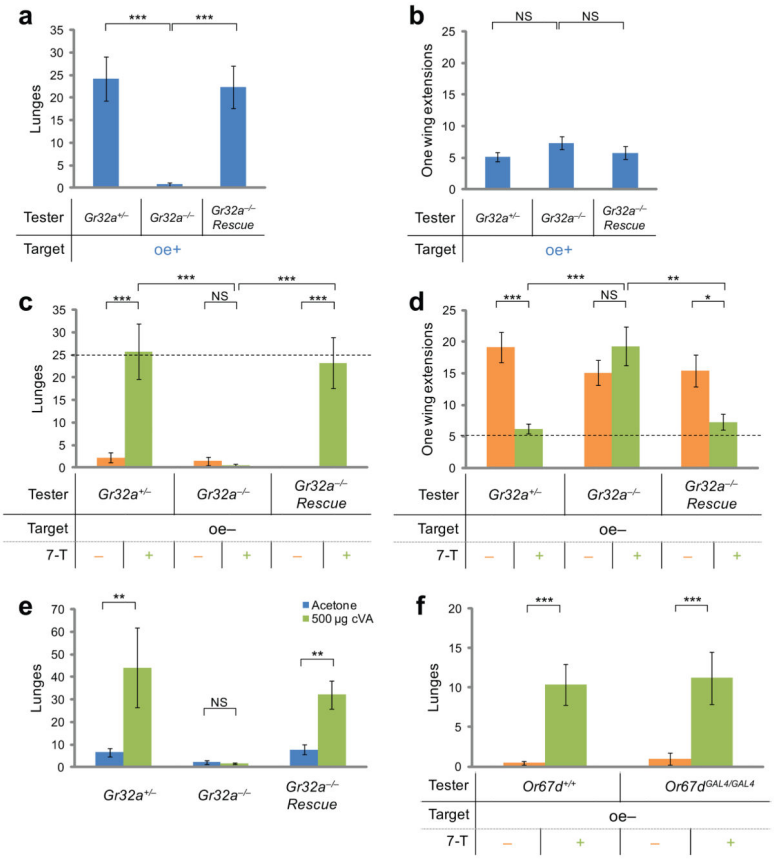

Figure 3. Gr32a mediates the behavioral effects of 7-T and permits the aggression-promoting effect of cVA

(a, b) Quantification of aggression (a) and courtship (b) performed by tester males of the indicated genotypes towards oe^{+} target males (n=26–28). (c, d) Quantification of aggression (c) and courtship (d) performed by tester males of the indicated genotypes towards oe^{-} target males (orange) or oe^{-} males perfumed with synthetic 7-T (green) (n=26–30). Dashed lines represent control levels of social behaviors (performed by *Gr32a*$^{+/-}$ testers towards oe^{+} targets (from Fig. 3a, b)). (e) Quantification of aggression performed by pairs of males of the indicated genotypes, in the presence of acetone alone (blue), or in the presence of 500 µg synthetic cVA (green) (n=18–20). Note the flies were group-housed prior to the behavioral assays to better reveal the effect of cVA (see Methods). (f) Quantification of aggression performed by tester males of the indicated genotypes towards oe^{-} target males (orange) or oe^{-} males perfumed with 7-T (green) (n=20). NS: p>0.05, *p<0.05, **p<0.01 and ***p<0.001.

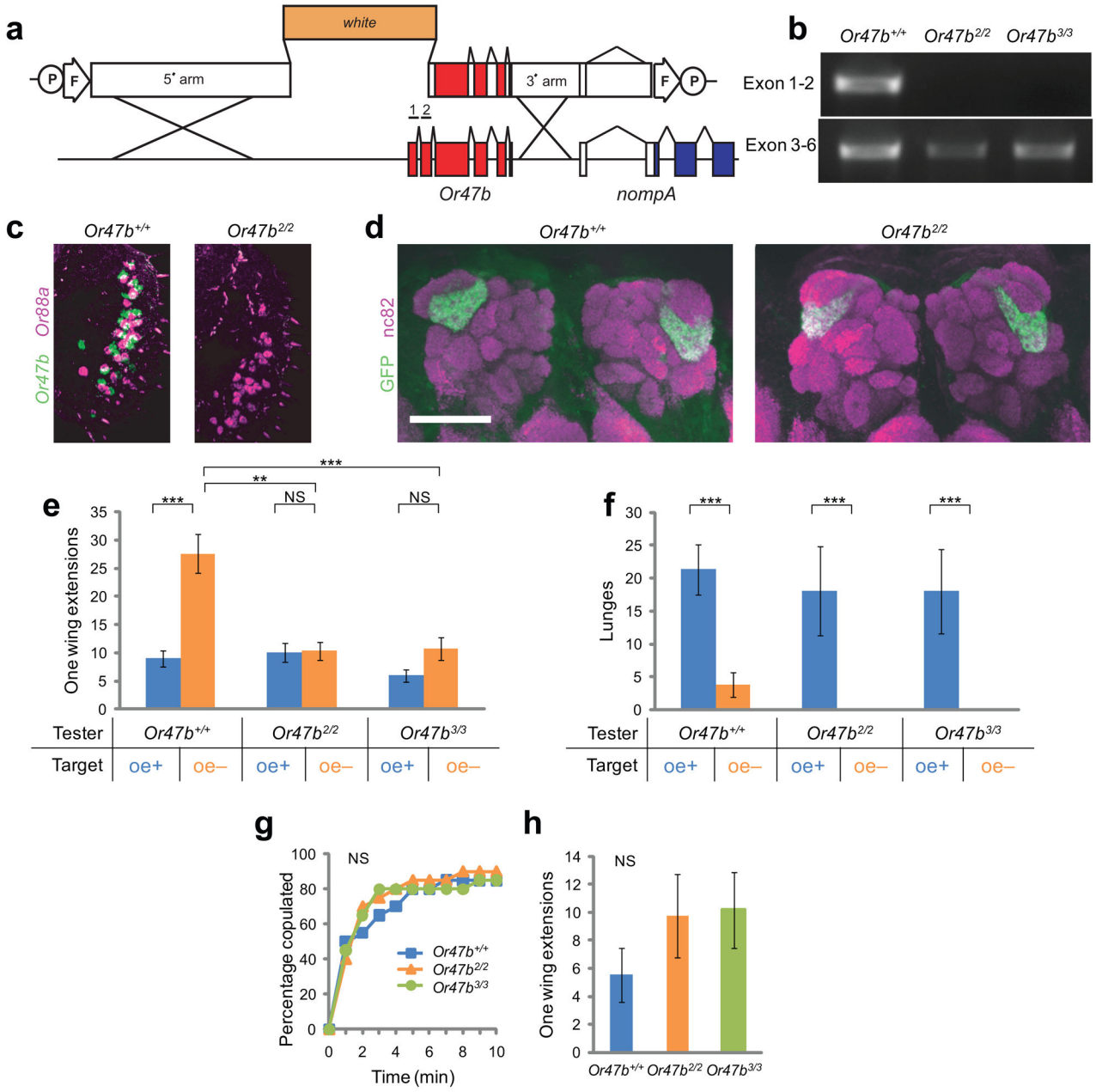

Figure 4. Or47b is required for elevated male-male courtship caused by depletion of male CHs

(a) Schematic illustration of the targeting construct (top) and site of homologous recombination (bottom) in *Or47b* locus. The flanking gene (*nompA*) included in the targeting construct is not disrupted. (b) PCR validation of two independently recovered mutant alleles lacking the first two exons of *Or47b*. (c) RNA *in situ* hybridization for *Or47b* (green) and *Or88a* (magenta). (d) Projections of Or47b^{+} ORNs to the VA1lm glomeruli visualized using *Or47b-GAL4; UAS-mCD8GFP* in flies of the indicated genotypes; nc82, neuropil counter-stain. Scale bar = 50 mm in (c, d). (e, f) Quantification of aggression (e) and courtship (f) performed by tester males of the indicated genotypes towards oe^{+} (blue) or oe$^{–}$ (orange) target males (n=20). (g, h) Cumulative latency to copulation (g; n=20) and

unilateral wing extension frequency (h; n=17–18) by males of the indicated genotypes towards virgin females. NS: $p>0.05$, *$p<0.05$, **$p<0.01$ and ***$p<0.001$.