GENETIC AND NEURAL REGULATION OF AGGRESSIVE BEHAVIOR IN *DROSOPHILA MELANOGASTER*

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This dissertation is dedicated to my grandma, who sadly passed away during my writing. She raised me up, and gave me her unreserved support and love.

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ABSTRACT

Aggression is an evolutionarily conserved behavior across the animal kingdom. Aggressive behavior among conspecifics is critical for the acquisition and defense of important resources including food, mates, and shelter, hence contributing to the survival and reproduction of animals. Therefore, it is of particular interest to understand how this behavior is regulated.

We use the fruit fly *Drosophila melanogaster* as a model system to understand the regulation of aggression. We identify *Cyp6a20*, a cytochrome P450, as a gene mediating the suppressive effect of social experience on the intensity of male-male aggression. Notably, *Cyp6a20* has been previously identified by profiling *Drosophila* strains subjected to genetic selection for differences in aggressiveness. Therefore our findings reveal a common genetic target for environmental and heritable influences on aggressiveness. Interestingly, *Cyp6a20* is expressed in a subset of non-neuronal support cells associated with pheromone-sensing olfactory sensilla, suggesting that olfactory pheromone(s) may contribute to the regulation of aggression. Consistent with this idea, we find that *cis*-11-vaccenyl acetate (cVA), a previously identified olfactory pheromone, promotes male-male aggression via a group of olfactory receptor neurons expressing Or67d.

Despite its robust behavioral effect, cVA is not required for baseline male-male aggression, and exogenous cVA does not induce male-female aggression, suggesting that sex specificity of male aggression is independent of cVA. Our subsequent studies show that the sex specificity of male social behaviors is determined by a different class of pheromones, named male cuticular hydrocarbons. Male flies perform significantly less

aggression and more courtship towards male flies lacking male CHs, both of which can be rescued by synthetic (*Z*)-7-tricosene (7-T), the most abundant male cuticular hydrocarbon. 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 cVA, but not vice versa. Furthermore, the increased courtship in the absence of male cuticular hydrocarbons is induced by pheromone(s) detected by an 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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Chapter 1

Introduction

Genetic and neural regulation of aggression in *Drosophila melanogaster*

Aggression is an evolutionarily conserved behavior across the animal kingdom. Conspecific aggressive behavior is critical for the acquisition and defense of important resources including food, mates, and shelter, hence contributing to the survival and reproduction of animals. Therefore, it is of particular interest to understand how this behavior is regulated.

Despite the accumulated progress in the past decades, the biology of aggression is incompletely understood. The remaining questions include but are not limited to: how do the environment and the internal state influences aggression? How does previous experience modulate animal's behavioral choice? What are the sensory modalities involved in the regulation of aggression, and how are they integrated? What is the genetic network underlying the development, initiation, and modulation of aggression? And what is the relationship between the connectivity/activity of various neuronal populations and aggression?

In our laboratory we use *Drosophila melanogaster* as a model system to address these unresolved questions regarding the regulation of aggressive behavior. In this chapter, I will summarize the current understanding of the aggressive behavior in the fruit fly, and will discuss the possible future research directions.

I: *Drosophila* as a model organism to study aggression: in retrospect

The first laboratory study of aggressive behavior in *Drosophila* can be traced back to almost a century ago. In a paper published in 1915 (Sturtevant, 1915), Alfred Sturtevant noted "*in such cases* [two males courting with one female] *they* [two males]

may sometimes be seen to spread their wings, run at each other, and apparently butt heads. One of them soon gives up and runs away. If the other then runs at him again within the next few minutes he usually makes off without showing fight". In this short yet landmark statement, Sturtevant not only characterized and classified several aggressive behaviors of male flies (now named "wing threat", "chasing", and "lunge" (Chen et al., 2002)), but also noticed two important intrinsic and extrinsic factors that regulated aggression (the presence of female (Chen et al., 2002); and the effect of losing a fight (Penn et al., 2010; Yurkovic et al., 2006). See Section II.). Taken together, this very first documentation already ensured the fruit fly a good model system to study the biology of aggression.

However, in the nearly 90 years after—although this tiny insect species became the workhorse of many branches of modern biology, including genetics, developmental biology, and neurogenetics—its potential as a model system to study aggression was long neglected. Evidently, there were only a handful of papers published between 1915 and 2002 on the topic of fly aggression. Nevertheless, important discoveries were still made in this "silent" period. Multiple sex-specific aggressive behaviors were better characterized, such as lunge and wing threat in males (Dow and von Schilcher, 1975; Jacobs, 1960) and head butt in females (Ueda and Kidokoro, 2002). A variety of extrinsic factors that influenced the aggressive behavior in flies, such as size (Hoffmann, 1987b; Partridge et al., 1987), social experience and age (Hoffmann, 1990; Ueda and Kidokoro, 2002) were investigated. The heritability of aggression/territoriality properties was also documented (Hoffmann, 1987a, 1988, 1991), implying that this behavior was encoded and regulated by the fly genome. Consistent with this idea, single-gene mutants that

showed altered motor pattern of aggression (Lee and Hall, 2000) or altered levels of aggression (Jacobs, 1978) were identified. As for a complementary approach beside the single-gene mutant studies, artificial selections of aggressive behavior were also conducted (Harshman and Hoffmann, 2000; Hoffmann, 1989). The selected hyper-aggression phenotype could be stabilized after multiple rounds of selection, further confirming the heritability of this behavior. Besides, from a neurobiological perspective, the functions of biogenic amines in fly aggression were investigated, primarily by using pharmacological manipulations (Baier et al., 2002). Last but not least, the correlations of aggressive behavior, territorial defense, and mating success (Dow and von Schilcher, 1975; Hoffmann, 1989) were reported, highlighting the ecological significance of this behavior.

A few outstanding issues, however, burdened the progress of aggression research in *Drosophila*. There had been no standardized protocol for aggression assays. Aggression assays utilized by different researchers differed in environmental conditions (temperature, humidity, illumination, size and shape of aggression arena, presence of food or not, duration of observation, etc.) as well as fly conditions (number, age, prior housing condition, etc.). Therefore it had been proven difficult to generalize the observations and conclusions made in one laboratory to others. Besides, although both single-gene mutations as well as artificial selections were shown to influence fly aggression, the potential of fly genetics (e.g., unbiased genetic/neuronal screens, enhancer/suppressor screens, mutation mapping, mosaic analysis, etc.) had not been fully explored. Ironically, the fruit fly, if only considered as a non-genetic model organism (i.e., for ecological, ethological, or pharmacological studies), is probably not the best system to

investigate the biology of aggressive behavior, due to the low levels of aggression in lab strains of fruit flies, a possible result of domestication (Dierick and Greenspan, 2006).

In 2002, one paper largely addressed the first outstanding issue. Edward Kravitz and colleagues (Chen et al., 2002) tried to standardize the behavioral setup (an elevated food cup with a decapitated female was placed in the center of a rectangular shaped aggression arena to attract male flies and to increase aggression) and the fly conditions (single-housed male flies to increase aggression; flies older than 3 days). More importantly, Chen et al for the first time described male aggressive behavior in *Drosophila* in a comprehensive way. They carefully recorded and categorized multiple types of aggressive encounters, and characterized their temporal relationship using Markov chain analysis. The same group described the female aggression in *Drosophila* in a similar way two years later (Nilsen et al., 2004). Most if not all subsequent studies on fly aggression more or less followed their protocols, making the observations and conclusions made in different laboratories more reliable and accessible. Ever since then, extensive studies to understand this evolutionarily conserved behavior in flies have been conducted from different perspectives. Aggression research therefore quickly moved into the neurogenetics era.

In the following sections, I will focus on three distinct yet related questions: first, the extrinsic/intrinsic factors that influence *Drosophila* aggression and the underlying mechanism; the genetic basis of this behavior; and the neural circuitry basis of this behavior.

II: Extrinsic and intrinsic factors that influence fly aggression

As a complex social behavior, aggression is influenced by numerous extrinsic and intrinsic factors. In this section I will review our up-to-date understanding of a few important aggression-regulating factors.

Size. Conspecific animals engage in aggression primarily to compete for resources like food, mates, and territory. The size of an animal in an aggressive encounter seems to correlate (one way or the other) with its outcome. Ary Hoffmann and colleagues noted that wild mating males, as well as flies raised in low density in laboratory conditions, had larger sizes, and these larger flies showed higher mating success and territorial dominance (Hoffmann, 1987b; Partridge et al., 1987). A later study, however, argued that 25 ºC raised, smaller flies were advantageous in aggression compared to 18 ºC raised larger ones. An alternative approach to examine the relationship of body size and aggression outcome is to look at the natural size variations. Flies of the same genetic background and raised under identical conditions show considerable body size variations, and such variations correlate well with the aggression outcome (bigger=winner) (Hoyer et al., 2008; Partridge and Farquhar, 1983). Strikingly, a size difference of as little as ~ 8% is sufficient to determine the winner of a fight (Hoyer et al., 2008).

Notably, all these foregoing studies were based on correlative observations rather than causative manipulations. Therefore it is difficult to know whether it is the size difference that determines the outcome of aggression, or whether the factors that influence the fly size (mating condition, housing density, temperature, and genetic polymorphism) also regulate aggression. Nevertheless, size is an important determination

factor of fly aggression, and should be taken into account when investigating the genetic and neural basis of this behavior.

Competition for resources. It is not surprising that the presence of resources (food or female flies) promotes male aggressive behavior (Chen et al., 2002; Jacobs, 1960). It is possible that specific cues released by the resource directly promote aggression by activating designated sensory neurons (e.g., food odor; sweet compounds in food; female-specific stripes on the abdomen; female pheromones). Alternatively, male flies may be able to abstract the sense of "resource" and escalate their aggressive behavior. It may be an interesting idea to see if the aggression-promoting resources like fly food, rotten fruits and female flies stimulate common sensory modality (e.g. sweet-sensing gustatory neurons?), and, if not, how distinct types of resources are conveyed by the sensory system and fed onto the central aggression circuitry (see Section IV). Lastly, it is worth noting that the aggression-promoting effect of resources can also be an indirect one, secondary to an aggregation-promoting effect.

Social experience. As in many other animal species (Day et al., 1982; Gallagher et al., 1972; Matsumoto et al., 2005), living with conspecifics greatly reduces aggression in male and female flies (Hoffmann, 1990; Ueda and Kidokoro, 2002; Wang et al., 2008). Such suppressive effect on aggression works autonomously through reducing aggressiveness towards opponent flies, rather than non-autonomously through evoking less aggression from other flies ((Ueda and Kidokoro, 2002), and also see Chapter 4). Also, this behavioral effect is reversible in the adult stage, suggesting social experience does not modulate aggression by altering the normal development of flies (Wang et al.,

2008). Attempts have been made to use this unique and robust behavioral effect to identify genes that underlie the regulation of fly aggression (Ueda and Wu, 2009; Wang et al., 2008). For example, *Cyp6a20*, a fly cytochrome P450 gene, was found to mediate the social suppression of male aggression (Wang et al., 2008), possibly by regulating pheromone sensitivity. And two genes, *Hyperkinetic* and *glutathione S-transferase-S1*, have been shown to mediate the social suppression of female aggression, via a mechanism possibly involving neuromuscular hyperexcitability and reactive oxygen species metabolism (Ueda and Wu, 2009).

Notably, social experience influences multiple fly behaviors besides aggression, including circadian rhythm (Levine et al., 2002), sleep (Ganguly-Fitzgerald et al., 2006), and courtship (Dankert et al., 2009). Several lines of evidence imply that the social regulation of fly behaviors involves chemosensory systems and pheromones (Ganguly-Fitzgerald et al., 2006; Kent et al., 2008; Krupp et al., 2008; Levine et al., 2002; Wang et al., 2008). However, a direct prove is still missing. Alternatively the physical interactions among conspecific flies during group housing may be involved. Also, whether social experience modulates these behaviors via a common mechanism or discrete mechanisms is an important question that remains to be investigated.

Prior aggressive experience. Besides living experience in a socially enriched environment, other types of social experience, especially prior antagonistic experience, greatly influence aggressive behavior. After one or several aggressive encounters, the socially defeated animals (subordinates) shift their behavioral choice from aggression to avoidance or surrender, which may contribute to the formation of social hierarchy (Huber

et al., 1997; Iwasaki et al., 2006; Siegfried et al., 1984; Yeh et al., 1996). In invertebrate species, several neuromodulators such as octopamine (OA) (Adamo et al., 1995; Hunt, 2007; Stevenson et al., 2005; Stevenson et al., 2000), dopamine (DA) (Stevenson et al., 2000), and serotonin (5-HT) (Yeh et al., 1996) have been implicated in the behavioral switch after social defeat. In *Drosophila*, subordinate males show similar behavioral switch in subsequent fighting (aggression→retreat/avoidance) (Penn et al., 2010; Yurkovic et al., 2006). It remains unclear, however, whether biogenic amines like OA/DA/5-HT are also involved in the behavioral switch in loser fruit flies.

Notably, in rodents, prior aggression experience not only modulates behavioral choice in subsequent fighting, but also influences a variety of non-aggressive behaviors, such as mating (D'Amato, 1988), performance in depression/anxiety assays (Bartolomucci et al., 2005), addiction (Ribeiro Do Couto et al., 2009), pain sensation (Siegfried et al., 1984), etc. Therefore, it is possible that social defeat induces a general "depressive" state in which multiple behaviors that rely on arousal and/or motivational state of animals are suppressed. It may be interesting to see if in fruit flies social defeat also has a general behavioral effect, and if we can better understand the relationship of social defeat, arousal states and human depressive disorders in this genetically tractable organism.

III: Identification of aggression-regulating genes in *Drosophila*

The first aggression-regulating genes in *Drosophila* were identified decades ago. M. E. Jacobs found that flies carrying mutant alleles of *black* or *ebony* genes, both of which were involved in β-alanine metabolism, showed altered aggression levels (Jacobs, 1978). Another class of genes, the sex determination hierarchy genes *fruitless* and *dissatisfaction* were also shown to influence fly aggression (Lee and Hall, 2000). Notably, Lee and Hall found that several alleles of *fruitless* and *dissatisfaction* mutant males exhibited distinct a motor pattern of aggression: "head-to-head interaction", which was soon found to be a typical female type aggressive posture (head butt) (Nilsen et al., 2004). Therefore, unlike *black* and *ebony*, *fruitless* and *dissatisfaction* regulate the sex-specific output of aggression in *Drosophila*.

Nevertheless, during this early stage, the attempts to identify aggression-regulating genes were still quite limited and sparse: mutants representing only 4 genes were characterized between 1915 and 2000! But since the "re-discovery" and detailed characterization of *Drosophila* aggression by Edward Kravitz and colleagues (Chen et al., 2002; Nilsen et al., 2004), the identification of aggression-regulating genes have exploded in the past decade. Here I will briefly review the recent progress.

Gene discovery from behavioral variations. One effective way to hunt for genes that regulate behaviors is to look for behavioral variations, naturally occurred or artificially established, and investigate the underlying genetic mechanism (Greenspan, 2003). As discussed in Section II, natural variations in aggression are present in the population of fruit flies. And multiple generations of artificial selection of aggressive

behavior could stabilize the natural variations (Greenspan, 2003; Hoffmann, 1989). So far, artificial selections of aggressive behavior have been used to identify aggression-regulating genes in two different laboratories (Dierick and Greenspan, 2006; Edwards et al., 2006). In both cases, for each round of the selection, male flies that showed high levels of aggression were selected and paired with random females to produce progeny used for the next round of selection. The hyper-aggressive phenotype could be stabilized after as short as 10 generations of selection (Dierick and Greenspan, 2006). Both laboratories used whole genome gene expression profiling to examine the differentially expressed genes between hyper-aggressive populations after generations of targeted selection vs. control populations (derived from either random selections (Dierick and Greenspan, 2006) or selections towards low aggression (Edwards et al., 2006)). As a result, a collection of aggression-promoting or -suppressing genes were identified (Robin et al., 2007).

A similar approach has also been applied to identify genes whose expression profiles might contribute to the natural variations of aggression in multiple inbred fly strains (Edwards et al., 2009b). Notably, the variations of aggression, either naturally occurred or artificially selected, may also be a result of genomic polymorphism rather than gene expression, which cannot be revealed by only profiling the gene expressions (e.g., microarray analysis). Quantitative trait loci (QTL) analysis was therefore performed as a complementary method to identify aggression-regulating genomic polymorphisms (Edwards and Mackay, 2009). Taken together, the above studies identified a few hundred of aggression-regulating genes that fell into highly diverse functional groups (Dierick and Greenspan, 2006; Edwards et al., 2009a; Edwards et al., 2009b; Edwards and Mackay,

2009; Edwards et al., 2006), underscoring the complex nature of aggression regulation in *Drosophila*. In spite of the progress, it is worth noting here that how these genes regulate aggression remains largely elusive.

Besides the heritable variations of aggression, environmental variations of aggression are also present in fly populations (Hoffmann, 1990; Wang et al., 2008). We also took the gene expression profiling approach to study how social experience suppressed male aggression. Strikingly, we found that *Cyp6a20*, a cytochrome P450 gene involved in the heritable variations of fly aggression after generations of artificial selection (Dierick and Greenspan, 2006), also mediated the social suppression of aggression within a single generation (Wang et al., 2008). The discovery of a single gene that mediates both the environmental and heritable influences on the same behavioral trait suggests a possible "nature vs. nurture" example: a gene involved in the regulation of a behavioral "state" (nurture) may be selected over generations to establish a behavioral "trait" (nature).

Gene discovery from genetic screens. Besides the behavioral variation-based gene discovery, *Drosophila* is highly amenable for identifying behavior-regulating genes by mutagenesis-based genetic screens, as first demonstrated by Seymour Benzer and colleagues (Benzer, 1967; Konopka and Benzer, 1971; Quinn et al., 1974). So far only one small-scale screen has been done to identify single gene mutations that give rise to aggression phenotypes in *Drosophila*. In this study, Trudy Mackay and colleagues screened 170 P-element mutant lines and identified 59 lines that exhibited increased or decreased aggressive behavior (Edwards et al., 2009a). Notably, in this study the

candidate mutant lines were not selected in an unbiased/random way, but were selected based on a gene expression profiling study of flies artificially selected for aggressive behavior (Edwards et al., 2006). Therefore the full potential of unbiased genetic screens to identify aggression-regulating genes has not been reached. The recently developed computer programs for automated behavioral analysis in flies (Dankert et al., 2009; Hoyer et al., 2008) may greatly facilitate large-scale, unbiased forward genetic screens (chemical mutagenesis or P-element based) for the discovery of aggression-regulating genes. Alternatively, RNA interference (RNAi) based (Dietzl et al., 2007; Ni et al., 2009; Ni et al., 2011) reverse genetic screens may also be performed to identify aggression-regulating genes. Notably, such reverse genetic screens could be done in a time- and cell-type-specific manner (Neely et al., 2010a; Neely et al., 2010b; Yapici et al., 2008), helping to elucidate how particular genes contribute to behavioral phenotypes.

IV: Sensory input and aggression

Fly aggression heavily relies on precisely presented sensory cues. These sensory cues either derive from the environment or from the opponent flies. On the one hand, food and the presence of females promote male-male aggression (Chen et al., 2002; Nilsen et al., 2004), suggesting sensory input from resources like food and female flies can influence aggression. On the other hand, a male fly never exhibits any aggressive posture when placed alone or when paired with a female (Dankert et al., 2009), suggesting the requirement of specific sensory cues from male opponents. Here I will

review how different sensory modalities influence fly aggression, primarily emphasizing on the detection of sensory cues from opponent male flies.

Olfactory system. The molecular and neuronal organizations of the olfactory system in *Drosophila* have been well understood (Vosshall and Stocker, 2007). And its function in fly social behaviors including courtship and aggression has become a remarked research topic in the past decade (Dickson, 2008; Montell, 2009; Vosshall, 2008). Olfactory input is required for male-male aggression, as mutant males flies lacking Or83b receptor, the co-receptor required for the normal function of ~ 70% olfactory receptor neurons (ORNs), show aggression deficit (Wang and Anderson, 2010). *cis*-11-vaccenyl acetate (cVA), a male-specific volatile pheromone previously shown to promote social aggregation and to suppress courtship (Ejima et al., 2007; Kurtovic et al., 2007; Xu et al., 2005), was found to greatly enhance male-male aggression via a group of ORNs expressing Or67d (Wang and Anderson, 2010), the cVA receptor (Ha and Smith, 2006; Kurtovic et al., 2007; van der Goes van Naters and Carlson, 2007). Although cVA detection via Or67d is not required for baseline male-male aggression, the activity of $Or67d^{+}$ ORNs is (at least partially) sufficient for aggression (Wang and Anderson, 2010). Whether there is additional olfactory pheromone(s) that regulate male-male aggression, and if so how it is detected, remains unknown.

Unlike most odorants, cVA is not directly detected by its olfactory receptor Or67d. Structural and electrophysiological evidence suggest that cVA is presented to the receptor by a cargo, an odorant binding protein named LUSH (Kim et al., 1998). In the absence of functional LUSH, cVA cannot activate $Or67d^{+}$ ORNs (Ha and Smith, 2006; Xu et al.,

2005). And mutant LUSH proteins that resemble its cVA-bound confirmation activate Or67d$^+$ ORNs in the complete absence of cVA and mimic its behavioral effects (Laughlin et al., 2008; Ronderos and Smith, 2010). In addition, cVA detection by Or67d$^+$ ORNs requires SNMP, a fly CD36 homolog working downstream of LUSH (Benton et al., 2007; Jin et al., 2008). Both LUSH and SNMP are not required for the detection of most fruit-derived odorants, suggesting their specific functions in pheromone (that is, cVA) detection. LUSH and SNMP may function to facilitate the delivery/transportation and hence the sensitivity of cVA. And conversely, they may also involve in the regulation of cVA sensitivity under different circumstances. It is also worth noting here that although LUSH and SNMP have been shown to be involved in cVA detection, there is no evidence that they are dedicated to the detection of this particular pheromone.

Or67d$^+$ ORNs express *fruitless*, a sex determination hierarchy gene that determines the sexual dimorphism of fly nervous system (Manoli et al., 2006) (See Section V.). The S exon of *fruitless* is sex-specifically spliced to produce functional Fru^M isoforms in males but not in females (Demir and Dickson, 2005). Males lacking Fru^M show courtship deficit and female-type aggression, and females ectopically expressing Fru^M exhibit (male-type) courtship and male-type aggression, suggesting that sex-specific splicing of *fruitless* is both necessary and sufficient to determine the "sex" of social behaviors in flies (Demir and Dickson, 2005; Manoli et al., 2005; Vrontou et al., 2006). Fru^M is expressed in ~ 2% of neurons in the male nervous system, including clusters of neurons in the brain and ventral nerve cord, as well as peripheral sensory neurons such as Or67d$^+$ ORNs (Kimura et al., 2005; Manoli et al., 2005; Stockinger et al., 2005). Therefore, it is of interest to understand whether and how the Fru^M-positive neurons are

involved in cVA detection and mediating its behavioral effects. Richard Axel and colleagues found that the direct downstream neurons of Or67d^{+} ORNs, the projection neurons (PNs) that innervated with DA1 glomerulus in the antennal lobe, were also Fru^{M}-positive (Datta et al., 2008). These DA1 PNs are cVA-responsive and they send Fru^{M}-dependent, sexually dimorphic projections to the lateral horn, where they innervate with downstream targets (Datta et al., 2008). Given cVA has distinct behavioral effects in males (courtship-suppressing (Kurtovic et al., 2007), aggression-promoting (Wang and Anderson, 2010)) vs. in females (receptivity-increasing (Kurtovic et al., 2007)), the sexually dimorphic projection patterns of DA1 PNs may have behavioral relevance.

Tracing further down into the cVA circuitry, Richard Axel and colleagues identified the third-order cVA-responsive neurons in the fly brain (Ruta et al., 2010). Strikingly, the third-order neurons located in the lateral horn also exhibit sexual dimorphism. Two clusters of third-order neurons, DC1 and LC1, are present only in males but not in females; and both clusters are Fru^{M}-positive (Ruta et al., 2010). These two clusters of neurons may mediate the male-specific cVA responses like courtship-suppressing and aggression-promoting. Notably, LC1 cluster neurons are inhibitory and DC1 neurons are excitatory (Ruta et al., 2010). There is therefore an attractive possibility that LC1 and DC1 neurons mediate cVA's suppressive (on courtship) and stimulatory (on aggression) effects in male flies, respectively. This may just be an over simplified hypothesis but it may worth testing experimentally.

Gustatory system. Contact-dependent, non-volatile pheromones are also involved in the regulation of fly social behaviors (Montell, 2009). One the one hand,

gustatory input from males suppresses male-male courtship. Eliminating all gustatory sensilla (Krstic et al., 2009), specific gustatory receptors (Miyamoto and Amrein, 2008; Moon et al., 2009), or male cuticular hydrocarbons (CHs) (Billeter et al., 2009; Savarit et al., 1999), the putative ligands of fly gustatory receptors (Ferveur, 2005; Lacaille et al., 2007), results in increased male-male courtship. (*z*)-7-tricosene (7-T), the most abundant male CH, has been shown to suppress both male-male and male-female courtship (Billeter et al., 2009; Lacaille et al., 2007). On the other hand, gustatory system is necessary for normal levels of male-male aggression. Genetic ablation of CH-producing oenocytes (Krupp et al., 2008) in males results in the elimination of male CHs and the decrease of male-male aggression (Fernández et al., 2010). We further showed that 7-T could restore aggression towards oenocyte-eliminated males in a manner dependent on one gustatory receptor Gr32a (Miyamoto and Amrein, 2008) (see Chapter 4). Therefore, a single male CH, 7-T, is sufficient to define appropriate male-male social interactions by suppressing and inducing male-male courtship and aggression, respectively. 7-T activates bitter-sensing gustatory receptor neurons (GRNs) (Lacaille et al., 2007). Consistently, Gr32a, the receptor required for the behavioral effects of 7-T, is expressed broadly in bitter-sensing GRNs (Weiss et al., 2011) and is required for the detection of a variety of bitter compounds (Lee et al., 2010). Given that fly bitter-sensing GRNs often co-express multiple gustatory receptors (Weiss et al., 2011) and that *Drosophila* only has limited ability to distinguish different bitter compounds (Masek and Scott, 2010), it is possible that 7-T simply tastes bitter to male flies, and that such a generic bitter taste is sufficient to determine appropriate male-male social behaviors. Alternatively, 7-T may activate a specific subset of GRNs (expressing specific combination of gustatory receptors; located

in specific organs; etc.) and exert its behavioral effects via a rather dedicated circuitry, like cVA does. Experiments to distinguish these alternatives remain to be conducted. Furthermore, the identity of downstream neurons that convey 7-T information to the central brain remains elusive. Analogous to the cVA circuitry studies, it is of interest to see if 7-T circuitry is also Fru^M-positive, and if similar trans-synaptic tracings can be performed to reveal the structure of 7-T circuitry (Datta et al., 2008; Koganezawa et al., 2009; Ruta et al., 2010).

The interplay between olfactory and gustatory systems. The olfactory and gustatory systems work in a hierarchical manner to define male-male social behaviors. On the one hand, the aggression-promoting effect of cVA/Or67d pathway is dependent on the presence of 7-T/Gr32a pathway but not vice versa (Chapter 4). On the other hand, in the absence of male CHs, the increased male-male courtship is blocked by a mutation in *Or47b* gene (Chapter 4), suggesting that the courtship-suppressing effect of gustatory pheromones is also dominant to the courtship-promoting effect of olfactory pheromone(s). The circuitry basis of their interactions is of particular interest.

The fact that Gr32a is required for the aggression-promoting effect of cVA suggests that 7-T circuitry may feed onto the cVA circuitry and regulate its activity. Strikingly, the male-specific DC1 cluster of third-order cVA neurons may receive gustatory information: DC1 dendrites interdigitate with axonal projections of presumable gustatory neurons located in the subesophageal ganglion (SOG) (Ruta et al., 2010). Consistently, a male-enriched cluster of Fru^M neurons (mAL neurons. See Section V.) project to lateral protocerebrum and SOG, suggesting a possible function of these

neurons to relay gustatory information to the central *fruitless* circuitry (Kimura et al., 2005), e.g., 7-T/Gr32a (Koganezawa et al., 2009). Therefore, it is possible that the third-order cVA-responsive neurons or their downstream neurons are modulated by gustatory input such as 7-T. Calcium imaging of cVA responses in cVA circuitry (e.g., in DC1 neurons) +/- 7-T stimulation (direct stimulation or neuronal activation by channelrhodopsin) may be the best way to test this hypothesis.

It is worth noting that the behavioral gating of cVA responses by 7-T/Gr32a pathway does not necessarily imply circuitry-level gating. It is also possible that certain amount of baseline aggression is required for the aggression-promoting effect of cVA. Alternatively, the gating mechanism may be indirect at circuitry level: neurons other than the lateral protocerebrum-SOG connection mediate the gating. Given Fru^M is both necessary and sufficient for male aggression (Vrontou et al., 2006), it may be a valid approach to screen for Fru^M-positive neuronal populations that mediate the behavioral gating of cVA response by 7-T (e.g., in a gain-of-function setting, whether activating a specific cluster of Fru^M-positive neurons permits cVA responses in the absence of 7-T/Gr32a) (von Philipsborn et al., 2011; Yu et al., 2010).

The circuitry basis of the interaction between male CHs and Or47b is another interesting question. Like Or67d, Or47b is expressed in Fru^M-positive ORNs (Couto et al., 2005; Fishilevich and Vosshall, 2005). It is therefore possible that some Fru^M-positive interneurons (like DC1 and LC1 neurons) mediate the suppressive effect of male CHs on Or47b pathway. It may be interesting to trace down the Or47b circuitry and examine its interaction with the gustatory system.

Visual, auditory, and mechanosensory systems. Besides the extensively studied chemosensory systems, non-chemosensory systems like vision, audition, and tactile sensation also influence fly social behaviors. Male flies exhibit much reduced aggression in dark, so do blind mutant flies, suggesting an indispensible role for vision in fly aggression (Hoyer et al., 2008). It is not clear, however, whether vision plays a sole permissive role in aggression (e.g., to locate the opponent fly), or rather a more constructive role (e.g., to detect a specific visual pattern that indicates an opponent fly). Notably, a subset of neurons in fly visual system (in medulla, lobula, and optic tubercle) is Fru^M-positive, implicating their possible roles in regulating fly social behaviors (Manoli et al., 2005; Stockinger et al., 2005). It is of interest to test if the Fru^M-positive visual circuitry plays a role in fly aggression.

Auditory and tactile systems are both involved in the regulation of courtship behavior in *Drosophila* (Ejima and Griffith, 2008; Gailey et al., 1986). It is not clear, however, whether these sensory systems play a role in aggression (Jonsson et al., 2011). Similarly, it will be interesting to examine if Fru^M-positive neurons in these sensory systems are involved in the regulation of both social behaviors (Manoli et al., 2005; Stockinger et al., 2005).

V: Central circuitry governing aggression

As discussed above, a great deal of effort has been put into the understanding of how different sensory systems regulate fly aggression. The various sources of

information collected by these sensory modalities are conveyed into the central brain, how they are integrated and are combined with various internal factors to determine appropriate behavioral responses. Here I will discuss the current understanding of the neural circuitry in the central nervous system (CNS) that regulates aggression in *Drosophila*.

Biogenic amines. Multiple biogenic amines including dopamine (DA), serotonin (5-HT), octopamine (OA), tyramine (TA), and histamine (HA) are present in the insect nervous system (Monastirioti, 1999), playing important roles in various developmental and physiological processes. The best-studied biogenic amine in insect aggression is OA. OA levels increase during agonistic interactions of male crickets *Gryllus bimaculatus* (Adamo et al., 1995), suggesting its aggression-promoting function. Consistent with this idea, pharmacological depletion of OA in crickets abolishes aggression, and topical application of OA agonists enhances it (Stevenson et al., 2005; Stevenson et al., 2000). In *Drsophila*, OA is present in ~ 100 neurons in the adult brain and OA-positive neurons send extensive projections over the fly brain (Busch et al., 2009). The function of OA in regulating fly aggression has been documented in recent years (Dierick, 2008; Potter and Luo, 2008). Genetic manipulations demonstrate that OA and OA-positive neurons are necessary and sufficient for aggression in the fruit fly (Hoyer et al., 2008; Zhou et al., 2008). Strikingly, restoring OA synthesis in as few as 2–5 neurons located in the SOG is sufficient to restore aggression in OA-depleted, *tyramine beta hydroxylase* mutant ($T\beta h^{-/-}$ (Monastirioti et al., 1996)) (Zhou et al., 2008). It was also reported that male flies with low/no OA activity showed increased tendency to court rather than to fight male flies (Certel et al., 2007). Such an effect of OA on male behavioral choice is likely mediated

by a separate group of OA-positive neurons (named ventral unpaired median (VUM) neurons), which co-express Fru^M (Certel et al., 2010; Certel et al., 2007). The relationship between OA's function to promote aggression and to modulate behavioral choice remains unclear. It is possible that two effects are mediated by Fru^M-negative and Fru^M-positive OA neurons, respectively (Certel et al., 2010). Four OA receptors have been identified in the fly genome (Evans and Maqueira, 2005). It is therefore also possible that different OA receptors mediate different behavioral effects of OA. Notably, OA has also been implicated in the regulation of non-aggressive behaviors in the fruit fly, including sleep (Crocker et al., 2010), appetitive learning (Schwaerzel et al., 2003), and ovulation (Lee et al., 2009). It is of interest to investigate how OA is engaged in various behaviors.

DA was also implicated in the regulation of fly aggression by pharmacological studies (Baier et al., 2002), although this conclusion so far has not been well supported by genetic manipulations that target all DA neurons (Alekseyenko et al., 2010). Notably, DA is involved in the modulation of a variety of behaviors, including courtship (Liu et al., 2008, 2009), arousal (Andretic et al., 2005; Lebestky et al., 2009), learning and memory (Claridge-Chang et al., 2009; Schwaerzel et al., 2003; Zhang et al., 2007), and locomotion (Friggi-Grelin et al., 2003; Kong et al., 2010). Therefore, it is possible that manipulating all DA neurons evokes global behavioral responses (especially those related to locomotion) which "mask" the aggression-modulating effect of DA. Selective labeling and manipulation of subsets of DA neurons may be a better way to examine the function of DA neurons in aggression.

There is also some controversy about the function of 5-HT in the regulation of fly aggression. An earlier study showed pharmacological manipulations of 5-HT levels (by feeding 5-HT precursor 5-HTP or 5-HT synthesis inhibitor pCPA) did not influence aggression (Baier et al., 2002). A later study, however, showed that 5-HTP feeding greatly enhanced aggression in certain lab-selected strains (Dierick and Greenspan, 2007). In this same study, Dierick and Greenspan found that activating both DA and 5-HT neurons promoted aggression while activating DA neurons alone did not, suggesting that 5-HT neurons were positive regulators of fly aggression (Dierick and Greenspan, 2007). Consistent with this observation, an independent study that manipulated only 5-HT neurons confirmed their positive influence on the escalation of aggressive behavior (Alekseyenko et al., 2010). It is worth noting that in different studies, different fly strains, 5-HT drugs, and *GAL4* drivers targeting different neuronal populations were used, which may contribute to the inconsistent observations. In addition, pharmacological manipulations also implicated the role for 5-HT receptors in fly aggression (Johnson et al., 2009).

***fruitless* circuitry.** As discussed earlier, *fruitless* specifies the sex of the fly nervous system (Manoli et al., 2006) via sex-specific splicing (Demir and Dickson, 2005). The male-specific isoform Fru^M is expressed in ~ 2% of neurons of the male nervous system, including clusters of neurons in the brain and ventral nerve cord, as well as peripheral sensory neurons including cVA neurons (Datta et al., 2008; Kimura et al., 2005; Manoli et al., 2005; Ruta et al., 2010; Stockinger et al., 2005). Given that Fru^M is both necessary and sufficient for male-type aggression and courtship (Demir and Dickson, 2005; Manoli et al., 2005; Vrontou et al., 2006), and that Fru^M-positive neurons are

interconnected to form an intact neural circuitry (Yu et al., 2010), it is reasonable to hypothesize that different subsets of Fru^M-positive neurons work in an coordinative manner to regulate fly social behaviors. The role of Fru^M-positive sensory neurons has been discussed in Section IV. Here I will mainly focus on the central *fruitless* circuitry.

Fru^M-positive neurons in male CNS show remarkable morphological dimorphism (Cachero et al., 2010; Kimura et al., 2005; Yu and Dickson, 2006; Yu et al., 2010). And these sexually dimorphic Fru^M neurons are likely to be involved in the regulation of male social behaviors including aggression and courtship. Some Fru^M-positive neuronal clusters are quantitatively different between male and female. For example, mAL (aDT2, aDT-b) cluster of Fru^M-positive neurons is composed of ~ 30 neurons in male vs. ~ 5 neurons in female (Kimura et al., 2005; Stockinger et al., 2005). mAL neurons also exhibit sexually specific projection patterns: male mAL neurons project both contra-laterally and ipsi-laterally, while female mAL neurons only project contra-laterally and show folk-like arborization patterns (Kimura et al., 2005). Both male and female mAL neurons project to lateral protocerebrum and SOG, suggesting a possible function of these neurons to relay gustatory information to the central *fruitless* circuitry (Kimura et al., 2005). One possibility may be that mAL neurons convey 7-T information to the central *fruitless* circuitry (Koganezawa et al., 2009). Given 7-T/Gr32a is required for male-male aggression (Chapter 4), it is of interest to test if Fru^M-positive mAL neurons play a role in aggression.

In addition to exhibiting sex-specific differences in cell numbers and/or branching patterns, some Fru^M-positive clusters are male-specific. For example, the P1 (or pMP4 or

pMP-e) cluster that is located in the lateral protocerebrum is only present in males (Cachero et al., 2010; Kimura et al., 2005; Yu et al., 2010). Masculinization of randomly labeled P1 neurons in female is sufficient to evoke male-type courtship behaviors (Kimura et al., 2005), and activation of randomly labeled P1 neurons in males triggers courtship (Kohatsu et al., 2011; von Philipsborn et al., 2011), suggesting that P1 cluster is involved in the regulation of male courtship behavior. It will be interesting to test whether P1 cluster neurons are also involved in the regulation of male-male aggression, especially the wing threat behavior, given that P1 neurons induce the courtship song production by wing vibration.

In addition to mAL and P1, multiple Fru^M clusters are male specific or show quantitative morphological differences between males and females (Cachero et al., 2010; Yu et al., 2010). Some of them have been shown to be involved in male courtship behavior (von Philipsborn et al., 2011). The characterization of these sexually dimorphic Fru^M clusters opens a way to systematically examine their functions in regulating male aggression. It is therefore of particular interest to conduct cluster-based (von Philipsborn et al., 2011; Yu et al., 2010) and/or random labeling-based (Kohatsu et al., 2011; von Philipsborn et al., 2011) screens to identify specific Fru^M neurons that regulate different aspects of aggression in male *Drosophila*.

VI: Concluding remarks and future directions

This is probably the best time ever to study the genetic and neural basis of a complex social behavior like aggression in *Drosophila melanogaster*. With the recent progress in standardized behavioral assays (Chen et al., 2002; Nilsen et al., 2004), automated behavioral analysis software (Dankert et al., 2009; Hoyer et al., 2008), genetic toolboxes (Dietzl et al., 2007; Ni et al., 2009; Ni et al., 2011), and neurobiological toolboxes (Hadjieconomou et al., 2011; Hampel et al., 2011; Lee et al., 2000; Luo et al., 2008; Pfeiffer et al., 2008; Pfeiffer et al., 2010; Potter et al., 2010; Ruta et al., 2010; Tian et al., 2009), it becomes possible to systematically identify and characterize the genetic as well as neural circuitry components of fly aggression. I foresee that the biology of aggressive behavior will soon emerge in the fruit fly.

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Chapter 2

A common genetic target for environmental and heritable influences on aggressiveness in *Drosophila*

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ABSTRACT

Environmental as well as genetic factors can modulate aggressiveness, but the biological mechanisms underlying their influence are largely unknown. Social experience with conspecifics suppresses aggressiveness in both vertebrate and invertebrate species, including *Drosophila*. We have searched for genes whose expression levels correlate with the influence of social experience on aggressiveness in *Drosophila*, by performing microarray analysis of head tissue from socially isolated (aggressive) vs. socially experienced (non-aggressive) male flies. Among ~200 differentially expressed genes, only one was also present in a gene set previously identified by profiling *Drosophila* strains subjected to genetic selection for differences in aggressiveness. This gene, *Cyp6a20*, encodes a cytochrome P450. Social experience increased *Cyp6a20* expression, and decreased aggressiveness, in a reversible manner. In *Cyp6a20* mutant, aggressiveness was increased in group-housed, but not socially isolated, flies. These data identify a common genetic target for environmental and heritable influences on aggressiveness. *Cyp6a20* is expressed in a subset of non-neuronal support cells associated with pheromone-sensing olfactory sensilla, suggesting that social experience may influence aggressiveness by regulating pheromone sensitivity.

INTRODUCTION

Aggression is critical for the survival and reproduction of many animal species (Kravitz and Huber, 2003; Loeber and Hay, 1997; Tecott and Barondes, 1996). Although aggression is an innate behavior subject to genetic influences, levels of aggressiveness are subject to environmental modifications as well. An important unanswered question is whether these influences act by independent, or shared, biological mechanisms. While genes underlying heritable differences in aggressiveness are beginning to be identified (Dierick and Greenspan, 2006; Edwards et al., 2006), very little is known about the molecular mechanisms underlying environmental influences on aggression.

Environmental influences on aggressiveness have been well-documented in a variety of animal models. Social status established by previous agonistic experience influenced subsequent aggression-related behavior in the crayfish (Yeh et al., 1996) as well as in crickets (Stevenson et al., 2005). Resident female Mediterranean fruit flies (*Ceratitis capitata)* located at a resource had a higher probability of defeating an intruder, suggesting that experience on a resource may increase aggressiveness (Papaj and Messing, 1998). Male fruit flies *(Drosophila melanogaster* and *Drosophila simulans)* raised at high density failed to successfully defend their territories against males raised at low density, an effect potentially related to differences in body size (Hoffmann, 1987b).

Social experience with conspecifics is one environmental influence on aggressiveness that is common to many species, including humans (Loeber and Hay, 1997). Socially isolated male mice are more aggressive than group-housed males (Matsumoto et al., 2005). Similar phenomena have been reported in the rat (Luciano and Lore, 1975), cichlid fish *(Haplochromis burtoni)* (Ferno, 1978) and other vertebrate

species. Effects of social experience on aggressiveness have also been described in invertebrates. Hoffman (Hoffmann, 1990) reported that male *Drosophila* held in isolation exhibited more aggressive behaviors, and required less time to establish their territories, than males held in groups, suggesting that social experience suppresses aggressiveness in the fruit flies. Analogous observations have also been reported for female *Drosophila melanogaster* (Ueda and Kidokoro, 2002).

Taken together, these data suggest that the effect of social experience on aggressiveness is shared among many species. However, the nature of the molecular mechanisms mediating this effect, and whether they are evolutionarily conserved, are poorly understood. Here we have used *Drosophila melanogaster*, a genetically tractable organism in which aggression has been well characterized (Chen et al., 2002; Hoffmann, 1987a; Skrzipek et al., 1979) to investigate the molecular basis of the influence of social experience on aggressiveness.

RESULTS

Group housing suppresses aggressiveness in a reversible manner

Flies raised in isolation ,after eclosion, are more aggressive than those raised in groups (Hoffmann, 1990). To quantify more easily this behavioral difference, we modified a fight chamber (Hoyer et al., 2008) to permit multiplex analysis of aggressiveness (Supplementary Fig. 1). A pair of male flies of similar age and social experience (raised in isolation immediately following eclosion, or in groups of 10 male flies, for 3 days prior to the test) was transferred into a fighting arena containing a small food patch. Consistent with earlier reports (Chen et al., 2002), we observed that lunging behavior, in which one fly rears up on its hind legs and charges the other fly, was the predominant form of aggression (Hoyer et al., 2008). We therefore counted lunges as a measure of aggressiveness, during a 20-minute observation period. Three different parameters were measured: 1) the fighting frequency, defined as the percentage of fly pairs that exhibited at least one lunge (Fig. 1A, B); 2) the lunging intensity, defined as the average number of lunges, calculated for all pairs that exhibited at least one lunge (Fig. **1C**); and 3) the average latency to the first lunge (Fig. 1D). Flies single-housed for 3 days exhibited a mean fighting frequency of ~50% (53.3±14.3%) during the 20 min observation period, while group-housed flies did not exhibit any lunges (Fig. 1A, $p<0.01$). A similar difference was observed between flies group- or single-housed for 6 days (but transferred to a new vial at day 3) (Fig. 1B, G→G vs. S→S, $p<0.05$). These data confirm that in flies, as in other animals including mice, social experience suppresses aggressiveness.

The effect of social experience on aggressiveness was reversible. When flies were single-housed for 3 days, followed by 3 days of group housing prior to the test, the fighting frequency was as low as that of flies group-housed for 6 days (Fig. 1B, G→G vs. S→G, $p>0.05$). Conversely, when flies were group-housed for 3 days, followed by 3 days of single housing, their aggressiveness was approximately as high as that of flies single-housed for 6 days (Fig. 1B, S→S vs. G→S, $p>0.05$). The median lunging intensity and latency among pairs exhibiting at least one lunge were not significantly different across all social conditions (Fig. 1C, D, $p>0.21$, $p>0.12$).

Cyp6a20 shows differential expression levels in single- vs. group-housed flies

To investigate the molecular basis of social influences on aggressiveness, we performed comparative gene expression profiling on heads from 6-day-old, group- vs. single-housed male flies. Using criteria of fold change>1.25 and $p<0.002$, we identified 141 probe sets that were up-regulated, and 48 that were down-regulated, in single- vs. group-housed males (Supplementary Tab. 1). The differentially expressed genes fell into diverse ontological and biological categories, including neurotransmitter metabolism, immunity and olfaction.

While this work was underway, a report appeared (Dierick and Greenspan, 2006) describing a microarray comparison, using head mRNA, between *Drosophila* strains selected for increased aggressiveness (AggrI and AggrII), and strains selected for decreased aggressiveness (NeutrI and NeutrII). Multiple differentially expressed genes were identified, allelic variation in which may underlie heritable differences in aggressiveness. To determine whether there is any commonality in the molecular

mechanisms through which aggressiveness is modified by heritable and environmental factors, we compared the differentially expressed genes identified in our social experience experiments, with those identified by selective breeding. Applying the same criteria (fold change>1.25; $p<0.002$), we identified *Cyp6a20*, a cytochrome P450 gene, as the only gene similarly regulated in both datasets. *Cyp6a20* was expressed at relatively lower levels both in AggrI&II vs. NeutrI&II (Dierick and Greenspan, 2006), and in socially isolated vs. group-housed flies. An independent microarray experiment identified many genes differentially expressed in flies selected for increased aggressiveness, but *Cyp6a20* was not among them (Edwards et al., 2006). However that study differed in several important details from the methods used in our own experiments, as well as in (Dierick and Greenspan, 2006), including the conditions under which flies were tested, and the criteria used to define aggressive behavior.

These data led us to further investigate the role of *Cyp6a20* in the effect of social experience on aggressiveness. We first confirmed the correlation between *Cyp6a20* expression levels, and social experience, using quantitative RT-PCR (qRT-PCR). *Cyp6a20* expression was almost 3-fold lower in flies single-housed for 3 days, than in group-housed flies of same age (Fig. 2A, $p<0.01$). Furthermore, in flies switched from single housing to group housing after 3 days, or vice-versa, the levels of *Cyp6a20* expression changed in parallel with, but in the opposite direction to, the changes in aggressiveness caused by these social manipulations (Fig. 2B). The fact that there is, on the one hand, a positive correlation between levels of *Cyp6a20* expression and social experience (Fig. 2A, B), and on the other hand, a negative correlation between social experience and aggressiveness (Fig. 1), suggested that *Cyp6a20* expression levels might

be negatively correlated with aggressiveness. Indeed, a plot of *Cyp6a20* mRNA levels vs. aggressiveness was well fit by a linear regression function, with a correlation coefficient = -0.96 (Fig. 2C).

Cyp6a20 mediates the suppressive effect of group housing on aggressiveness

If *Cyp6a20* plays a role in mediating the effect of social experience, then flies deficient in *Cyp6a20* should exhibit higher fighting frequencies under group- but not single-housing conditions. Indeed, group-housed flies bearing a homozygous P-element insertion in the *Cyp6a20* locus showed a significantly higher fighting frequency than group-housed $Cyp6a20^{+/-}$ flies (Fig. 3A, gray bars, *Cyp6a20-/-* vs. *Cyp6a20+/-*, $p<0.05$), while there was no significant difference between these genotypes under single housing conditions (Fig. 3A, white bars, *Cyp6a20-/-* vs. *Cyp6a20+/-*, $p>0.05$). Furthermore, the fighting frequency of group-housed $Cyp6a20^{-/-}$ mutant flies was as high as that of single-housed $Cyp6a20^{-/-}$ mutant flies, while heterozygous $Cyp6a20^{+/-}$ flies (like wild-type Canton-S flies) showed a significantly reduced fighting frequency under group housing conditions (Fig. 3A). There was no statistically significant difference in locomotor activity between $Cyp6a20^{+/-}$ and $Cyp6a20^{-/-}$ flies under group housing conditions, nor between single- vs. group-housed $Cyp6a20^{+/-}$ flies, as measured by the total distance traveled during a 20-minute filming period (Fig. 4A). Furthermore, $Cyp6a20^{-/-}$ flies exhibited normal odor-guided behavior (Fig. 4B), and normal courtship behavior towards wild type virgin females (Fig. 4C-F), arguing that the mutation in *Cyp6a20* does not cause general deficits in olfaction or social behavior.

To confirm that the selective increase in aggressiveness under group housing conditions was indeed caused by the P-element insertion in the *Cyp6a20* locus, we tested the *Cyp6a20* insertion over a deficiency spanning the *Cyp6a20* gene, *Df(2R)BSC11* (Dierick and Greenspan, 2006). Like $Cyp6a20^{-/-}$ flies, $Cyp6a20^{Df/-}$ mutant flies showed a significantly higher fighting frequency than $Cyp6a20^{Df/+}$ hemizygous flies under group housing conditions (Fig. 3A, gray bars, *Df(2R)BSC11/+* vs. *Cyp6a20/Df*, $p<0.05$), while there was no significant difference between these genotypes under single housing conditions (Fig. 3A, white bars, *Df(2R)BSC11/+* vs. *Cyp6a20/Df*, $p>0.05$). In addition, the fighting frequency of $Cyp6a20^{Df/-}$ flies under group housing conditions was as high as that under single housing conditions (Fig. 3A). There was no statistically significant difference in locomotor activity between $Cyp6a20^{Df/+}$ and $Cyp6a20^{Df/-}$ flies under group housing conditions, or between group- vs. single-housed $Cyp6a20^{Df/+}$ flies (Fig. 4A). The lunging intensity and latency remained unchanged in all the four genotypes (Fig. 3B, C, $p>0.11$; $p>0.08$). Previous experiments have shown that the levels of *Cyp6a20* mRNA in $Cyp6a20^{-/-}$ and $Cyp6a20^{Df/-}$ flies are only 8%-15% of those in $Cyp6a20^{+/-}$ and $Cyp6a20^{Df/+}$ flies (Dierick and Greenspan, 2006). Taken together these data suggest that the phenotype of the P-element insertion indeed reflects a reduction in *Cyp6a20* expression or function, although rescue experiments will be required to formally confirm this. Thus, in flies with reduced levels of *Cyp6a20* expression, group housing is much less effective in suppressing aggressiveness (for more detailed analysis, see Supplementary Fig. 2). These genetic data, when taken together with the observation that *Cyp6a20* mRNA levels are up-regulated by group housing (Fig. 2A, B), suggest that *Cyp6a20* mediates the effect of social experience to suppress aggressiveness (Fig. 3D).

Cyp6a20 is specifically expressed in a subset of olfactory sensory organs

As an initial step towards investigating the mechanism of action of *Cyp6a20*, we investigated where the gene is expressed, using an enhancer trap line, *P[GawB]NP2593*, in which *Gal4* is integrated into the *Cyp6a20* locus. In *P[GawB]NP2593/UAS-mCD8GFP* adult flies, the reporter was expressed in the antennae and maxillary palps, the two main *Drosophila* olfactory sensory organs (Fig. 5A, B). Scattered GFP signal was also seen in the brain (data not shown). Thus, like other P450 genes described previously in *Drosophila* (Wang et al., 1999), *Cyp6a20* appears antennal-enriched. We wished to verify that the Gal4-targeted GFP expression faithfully recapitulated the expression pattern of endogenous *Cyp6a20* mRNA. However the small number and inaccessibility of GFP^+ cells in the antennae precluded a comparison of *Cyp6a20* mRNA levels between GFP^+ and GFP^- cells, and *Cyp6a20* expression was undetectable by in situ hybridization (not shown). We therefore examined the expression of *Cyp6a20* mRNA in larvae, where strong GFP expression was observed specifically in the salivary gland of *P[GawB]NP2593/UAS-mCD8GFP* specimens (Supplementary Fig. 3A). RT-PCR experiments performed on larval tissues confirmed that *Cyp6a20* transcripts were enriched in salivary gland (Supplementary Fig. 3B). The fact that the *P[GawB]NP2593* insertion in *Cyp6a20* correctly reports expression in larval tissues makes it reasonably likely that the same holds true for the adult, although further experiments will be required to confirm this.

Intriguingly, the GFP^+ cells in the adult antennae were preferentially if not exclusively associated with trichoid sensilla, which are thought to be involved in

pheromone detection (van der Goes van Naters and Carlson, 2007). Such trichoid sensillar-specific expression has been described for two P450 genes in the moth *Mamestra brassicae* (Maibeche-Coisne et al., 2004a), but has not been previously reported in *Drosophila*. More surprisingly, while it is often assumed that antennal-specific P450 enzymes are expressed by olfactory receptor neurons, GFP^+ cells in both the antennae and palps did not coexpress ELAV, a neuronal marker (Robinow and White, 1988) (Fig. 5C, D). However a subset of GFP^+ cells co-expressed LUSH (Fig. 5E, F), an odorant binding protein that marks a subpopulation of non-neuronal support cells (Kim et al., 1998). Thus, *Cyp6a20* is a P450 gene specifically expressed in a subset of olfactory sensory support cells.

DISCUSSION

Both genes and environment can influence aggressiveness, However it has not been clear whether there is a commonality to the underlying biological mechanisms has not been clear. Using *Drosophila* as a model system, we show that an evolutionarily conserved environmental influence on aggressiveness, social experience, is associated with changes in gene expression. Detailed analysis of one of the regulated transcripts, *Cyp6a20*, indicates that it is up-regulated by social experience, in a manner that correlates with the effects of social experience to suppress aggressiveness. Genetic experiments confirm that *Cyp6a20* is a negative regulator of aggressiveness (Dierick and Greenspan, 2006), but reveal that its influence is only observed under conditions of group housing, where its expression is relatively higher. These data suggest that *Cyp6a20* is required to mediate the effect of group housing to suppress aggressiveness. *Cyp6a20* was the only gene in our dataset in common with a set identified in an independent expression profiling analysis of *Drosophila* populations selected for differential levels of aggressiveness (Dierick and Greenspan, 2006). Taken together, these data suggest that *Cyp6a20* represents a common genetic target of heritable and environmental influences on aggressive behavior in fruit flies. (Fig. 6C) Whether *Cyp6a20* is the only such target remains to be determined.

Social experience influences aggressiveness by regulating gene expression

Prior social experience with conspecifics influences numerous aspects of animal behavior. Social isolation causes behavioral abnormalities in rodents, including anxiety and hyper-aggressiveness (Barrot et al., 2005; Champagne and Curley, 2005; Matsumoto

et al., 2005). *Drosophila melanogaster* reared as groups exhibit circadian rhythm coherence (Levine et al., 2002), and longer periods of day-time sleep (Ganguly-Fitzgerald et al., 2006). Social experience has also been shown to regulate courtship behavior in fruit flies (Svetec and Ferveur, 2005). In cichlid fish, social interactions can regulate the brain expression of genes encoding neuropeptides (White et al., 2002) and steroid hormone receptors (Burmeister et al., 2007), but it has been difficult to extend such observations from correlation to causality. Here we have demonstrated that extended male-male social interactions regulate gene expression in *Drosophila*, a system affording facile genetic manipulations. Recent studies have shown that rapid-onset changes in gene expression accompany male-female courtship in *Drosophila* (Carney, 2007). (We have not yet examined the influence of male-female interactions in group housing, on aggressiveness.) Further investigation of these genes may lead to a more comprehensive understanding of the effect of social experience on animal behaviors.

Under our stringency conditions, we identified 141 probe sets exhibiting higher expression in socially isolated than in group-housed flies, and 48 probe sets exhibiting higher expression under group-housing conditions. Since aggressiveness is higher in socially isolated flies (Hoffmann, 1990), genes in the first category are candidate positive regulators of aggressiveness, while those in the second category are candidate negative regulators. Loss-of-function mutations in candidate negative regulators should increase aggressiveness, as shown previously for *Cyp6a20* mutants (Dierick and Greenspan, 2006). The present analysis confirms this, but reveals that flies homozygous for a hypomorphic allele of *Cyp6a20* only show increased aggressiveness under group-housing conditions.

Interestingly, flies bearing mutations in two other candidate negative regulators of aggressiveness, which we identified in a similar analysis using a different fly strain, exhibited increased aggressiveness under single-housed conditions, but their aggressiveness could still be suppressed by group housing (L.W. and D.J.A., unpublished observations). These data suggest that there are at least two classes of genes that negatively regulate aggressiveness, and whose expression levels are relatively higher in group-housed compared to single-housed flies: (A) genes such as *Cyp6a20*, an hypomorphic allele of which overrides the effect of group-housing to suppress aggressiveness, but which does not increase aggressiveness under single-housed conditions (Fig. 3A, D, Class II); and (B) genes such as those identified in our second screen, hypomorphic mutations in which cause increased aggressiveness under single-housed conditions, but not under group-housed conditions (Fig. 3D, class I). In principle, a third category of negative regulators may promote constitutively increased aggressiveness when mutated, under both single- and group-housing conditions, but we have not yet identified exemplars of this class. Recent studies have implicated serotonin, octopamine and neuropeptide F in the control of aggressiveness in *Drosophila*. Genes related to these neuromodulatory pathways were not among those identified in our screen (Baier et al., 2002; Certel et al., 2007; Dierick and Greenspan, 2007; Hoyer et al., 2008).

Environmental and heritable influences on aggressiveness

Genes can influence behavior through both polymorphic variation, on which natural selection can act, and by environmentally regulated changes in expression that occur within the lifetime of an individual. For example, naturally occurring

polymorphisms in the *foraging* gene, which encodes a guanosine 3', 5'-monophosphate (cGMP)-dependent protein kinase (PKG), cause modifications of feeding behavior in *Drosophila melanogaster* (Osborne et al., 1997), while developmental changes in the expression of its honey bee ortholog, *Amfor,* modulate feeding behavior during the life history of single individuals (Ben-Shahar et al., 2002). It is not yet known whether naturally occurring polymorphisms in *Cyp6a20* itself, or rather in genes that encode upstream regulators of its expression, underlie the differences in *Cyp6a20* transcript levels between the Neutr and Aggr strains selected in (Dierick and Greenspan, 2006). Nevertheless our results, taken together with the genetic selection experiments in (Dierick and Greenspan, 2006), identify a common genetic target of environmental and heritable influences on aggressive behavior within a single species (Fig. 6).

Previous studies have shown that increased aggressiveness promotes enhanced mating success (Dow and Schilcher, 1975). This raises the question of why, if aggressive behavior provides a general selective advantage, the ability of social experience to suppress aggressiveness is not eventually lost over many generations, and replaced by constitutively aggressive populations. One explanation is that there may be positive selection for the ability of social experience to suppress aggressiveness. For example, under conditions where food resources are scarce and flies tend to feed in groups, individuals engaged in ongoing aggressive activity, despite this enriched social experience, might divert their energy and attention from feeding and reproductive behavior, thereby reducing their likelihood of reproductive success.

Control of aggressiveness by social regulation of cytochrome P450s

The fact that social experience suppresses aggressiveness in many species leaves open the question of whether the underlying molecular mechanisms are also conserved. Although *Cyp6a20* does not have a clear vertebrate ortholog, and appears to function in an insect-specific olfactory pathway (see below), it encodes a cytochrome P450, which encompasses a large family of proteins with diverse enzymatic activities (Robin et al., 2006). In vertebrates, one member of this family is aromatase, which converts testosterone to estrogen and is required for inter-male aggressiveness (Matsumoto et al., 2003; Toda et al., 2001). Interestingly, the expression of brain aromatase has been shown to be regulated by social experience and other environmental influences (Black et al., 2005; Soma et al., 2003). While flies lack testosterone, it is interesting that, in addition to *Cyp6a20*, 5 additional cytochrome P450 genes exhibited significant differential expression, by our criteria, between single housing vs. group housing conditions (Supplementary Tab. 1). Thus, the general role of aromatases in mediating environmental influences on aggressiveness may be conserved, even if the pathways in which they act are not.

The antennal-specific expression of *Cyp6a20*, taken together with its up-regulation by group housing and its functional role as revealed by genetics, suggests that social experience may cause changes in olfactory sensitivity, via regulation of *Cyp6a20*. Consistent with this interpretation, previous studies have shown that the effect of social experience on male-male interactions requires pheromonal perception (Svetec and Ferveur, 2005). Whether changes in *Cyp6a20* expression caused by social experience indeed influence pheromonal sensitivity, and if so in what direction, are not yet clear. Pharmacologic inhibition of antennal-enriched cytochrome P450's eliminates pheromone

sensitivity in some insects (Maibeche-Coisne et al., 2004b), suggesting a requirement for these proteins for maintaining olfactory sensitivity. If *Cyp6a20* were a positive regulator of pheromone sensitivity, then its increased expression under group-housing conditions might enhance sensitivity to an aggression-suppressing pheromone, resulting in a lower level of aggressiveness. Such a model would be consistent with the suggestion that inhibitory pheromones are used to suppress male-male interactions (Svetec and Ferveur, 2005). Alternatively, as suggested previously (Dierick and Greenspan, 2006) *Cyp6a20* may function to decrease sensitivity to an aggression-promoting pheromone. Distinguishing these hypotheses will require identification of the relevant pheromones and their functional role in aggression.

METHODS

Fly stocks and rearing conditions.

All fly stocks were reared in plastic vials containing yeast, corn syrup and agar medium, at 25°C, 60% humidity and 12h-light: 12h-dark cycle. Newly eclosed males were reared either individually (single housing) or at 10 flies (group housing) per vial [2.4 cm (Diameter) x 9.4 cm (Height)] for 3 or 6 days before performing the behavioral assay. Wild-type Canton-S (CS) flies were used for all experiments unless otherwise indicated. *Cyp6a20*$^{-/-}$ was introgressed into the CS background from y^1w^{67c23}; *P{y[+mDint2] w[BR.E.BR]=SUPor-P}KG04665* as described (Dierick and Greenspan, 2006). *Cyp6a20*$^{+/-}$ flies were generated by crossing *Cyp6a20*$^{-/-}$ males with Canton-S females. *Cyp6a20*$^{Df/+}$ *(Df(2R)BSC11/+)* flies were generated by crossing *Df(2R)BSC11/SM6a* males with Canton-S females. *Cyp6a20*$^{Df/-}$ *(Cyp6a20/Df)* flies were generated by crossing *Df(2R)BSC11/SM6a* males and *Cyp6a20*$^{-/-}$ females. P[GawB]NP2593 flies were from Drosophila Genetics Resource Center, Kyoto Institute of Technology, Japan.

Aggression assay

A multiplex aggression apparatus containing 5 arenas was constructed as illustrated in Supplementary Fig. 1. Two males of the same age and social experience, but from different vials, were introduced into each well by gentle aspiration without anesthesia. The behavior of 5 pairs was video-captured for 20 minutes and analyzed manually, by counting the number of lunges in each arena. Lunging behavior was defined as previously described (Chen et al., 2002). The temperature and humidity of the

apparatus was set to ~25°C and ~40%, respectively. Aggression assays were performed between 5pm and 12am.

Microarray analysis

Three biological replicates were performed for both single housing and group housing conditions. For each replicate, 20 heads were isolated from 6-day-old, single-housed or group-housed male flies that were collected and frozen at 5pm-6pm on different days. Total RNA was prepared using Trizol as described (Guan et al., 2005). The following steps, including RNA quality test, reverse transcription, cRNA labeling, fragmentation and hybridization (Affymetrix *Drosophila* Genome Array 2.0) were performed by the Millard and Muriel Jacobs Genetics and Genomics Laboratory at the California Institute of Technology. The raw data from all arrays are available online at http://www.ncbi.nlm.nih.gov/project/geo under series GSE6994.

Quantitative RT-PCR

Four biological replicates were performed for each group indicated in Fig. 2. For each replicate, 20 heads were isolated from flies which were collected and frozen at 5pm-6pm on different days. Total RNA was prepared using Qiagen RNeasy Micro Kit. For each biological replicate, three RT-PCR (technical) replicates were performed.

Other behavioral assays

Locomotor activity of flies was measured using a customized program written in Matlab (Dankert et al., in preparation). For each measurement, a pair of 6-day-old male

flies was introduced into a fighting arena with a food patch and agar, videotaped for 20 minutes and analyzed. To facilitate the automatic tracking of fly trajectories, the fighting arena used here was square-shaped instead of round-shaped as Supplementary Fig. 1. For food trap assay, male flies were group-housed (10 flies/vial) for 5 days and wet-starved for an additional day on 1% agar medium before the test. On the day of the test, 10 flies were carefully aspirated into a plastic cylinder with the same dimensions as the fighting arena (Supplementary Fig. 1), containing two odor traps (one food+ and one food-). The trap was made by inserting a P1000 pipette tip into a 5mL glass serum bottle (1.6cm (Diameter) x 3.4cm (Height)), containing 1ml of standard fly food (food+), or, as a control, 1% agar (food-). After two hours, the number of flies trapped in each trap was counted and analyzed. For courtship assay, male flies were raised at 10 flies/vial for 5-6 days before testing. On the day of the test, one male fly of a given genotype, and a wild-type (C-S) virgin female of a similar age were carefully aspirated into a square-shaped fighting arena, and videotaped for 30 minutes. Fly pairs that did not perform copulation were not included in the analysis. Three parameters were analyzed: courtship latency (the latency to the first courtship behavior exhibited towards the female), copulation latency (the latency of copulation) and courtship index (the percentage of time spent on courtship, including copulation, during the first 10 minutes of videotaping).

Statistical analysis of behavioral data

For two-group comparisons, the student's t test (Fig. 4D, 4E, 4F) or Mann-Whitney U test (Fig. 1A) were performed for parametrically or non-parametrically distributed data. For comparisons of more than two groups, ANOVA (Fig. 1B, 3A, 4A)

or Kruskal-Wallis ANOVA (Fig. 1C, 1D, 3B, 3C, 4B) was performed for parametrically or non-parametrically distributed data, respectively. We used Student-Newman-Keuls test (Fig. 3A) or Dunnett's C test (Fig. 1B, 4A, 4B) as a *post hoc* test following ANOVA, to identify significantly different groups, with or without the assumption of homogeneity of variance, respectively. Significance levels for ANOVA and Kruskal-Wallis ANOVA were set to 0.05. Bar graphs are used to illustrate comparisons of means, with error bars representing s.e.m.. Boxplots are used to illustrate comparisons of medians, with the lower and upper edges of the boxes representing the 25% and 75% quantiles, respectively, and the whiskers representing the 5% and 95% quantiles. The only exception was Fig. 1A, in which a bar graph (mean±s.e.m.) is used for illustrative purposes, while the statistical comparison was made between medians. This was done for the consistency with the other "Fighting frequency" graphs (Fig. 1B, 3A).

Statistical analysis of microarray and quantitative RT-PCR data

Array data were analyzed using the Rosetta Resolver platform through the default processing pipeline, including normalization, grouping and inter-group comparison. A fold-change of 1.25 and a P-value of 0.002 were used to identify differently expressed genes (Supplementary Tab. 1). For quantitative RT-PCR, Student's t-test was performed for the comparison between two normally distributed data sets (Fig. 2A). ANOVA followed by Student-Newman-Keuls *post hoc* test was used for the comparison between four normally distributed data sets with homogeneity of variance assumed (Fig. 2B).

Immunohistochemistry

Whole mount antibody staining was adapted from (Manoli et al., 2005). 3-6 days old male *P[GawB]NP2593/UAS-mCD8GFP* flies were anesthetized and dissected in PBS. Antennae (both 2nd and 3rd segments) and palps were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature (RT), washed 2X10 min in PBS, incubated in PBS containing 5% Triton X-100 (5% PBT) for 5 min, washed 3X10 min in 0.3% PBT, blocked 1h in 0.3% PBT containing 5% heat inactivated normal goat serum (0.3% PBT/S), and incubated with primary antibody in 0.3% PBT/S overnight at 4°C. On the following day, samples were incubated in 0.3% PBT/S at RT for 1h after 3X10 min wash in 0.3% PBT. Samples were then incubated with secondary antibody and TOPRO-3 in 0.3% PBT/S for 2h at RT in dark, washed 3X5 min in 0.3% PBT, mounted in Vectashield (Vector Labs), and imaged on confocal microscope (Leica).

Antibody staining of frozen sections was performed exactly the same as described in (Vosshall et al., 2000). 3-6 days old male *P[GawB]NP2593/UAS-mCD8GFP* flies were anesthetized, aligned using Martin Heisenberg fly collar, mounted in frozen Tissue Tek OCT, sectioned at 14μm, fixed in 4% paraformaldehyde in PBS at RT for 7 min, washed 2X10 min in PBS, penetrated in 0.1% PBT for 30 min, blocked in 0.1% PBT/S for 30 min, and incubated with primary antibodies in 0.1% PBT/S overnight at 4°C. On the following day, slides were washed 3X10 min in 0.1% PBT, blocked in 0.1% PBT/S for 30 min, incubated with secondary antibodies and TOPRO-3 in 0.1% PBT/S for 2h in dark, washed 3X5 min in 0.1% PBT, mounted in Vectashield, and imaged on confocal microscope (Leica).

Antibodies were used in dilution as followed: rabbit anti-GFP (Invitrogen, 1:800), chicken anti-GFP (Chemicon, 1:300), rabbit anti-LUSH (D. P. Smith, 1:20), rat anti-

ELAV (DSHC 7E8A10, 1:10), Alexa488/Cy3-conjugated secondary antibodies (Molecular Probes, 1:500), TOPRO-3 (1:2000).

ACKNOWLEDGEMENTS

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FIGURES

Figure 1. Social experience influences *Drosophila* aggressiveness

Upper diagrams illustrate experimental manipulations. (A) Mean fighting frequencies in 3-day-old, single-housed and group-housed flies (mean±s.e.m. n=6 groups each containing 5 fly pairs. **$p<0.01$). (B) Mean fighting frequencies in 6-day-old, S→S, G→S, G→G and S→G flies (mean±s.e.m. n=6 groups each containing 5 pairs. Significant differences ($p<0.05$) are indicated by letters above bars). (C) Median lunging intensities of 3-day-old, single-housed flies, and 6-day-old, S→S, G→S, G→G and S→G flies (n=16, 25,19,1,2 pairs, respectively; N.S., not significantly different ($p>0.05$)). (D) Median lunging latencies of 3-day-old, single-housed flies and 6-day-old, S→S, G→S, G→G and S→G flies (n=16,25,19,1,2 pairs, respectively; N.S. ($p>0.05$)). Comparisons between groups were made using the Mann Whitney U test (A), ANOVA followed by *post hoc* test (B) and Kruskal-Wallis ANOVA (C, D).

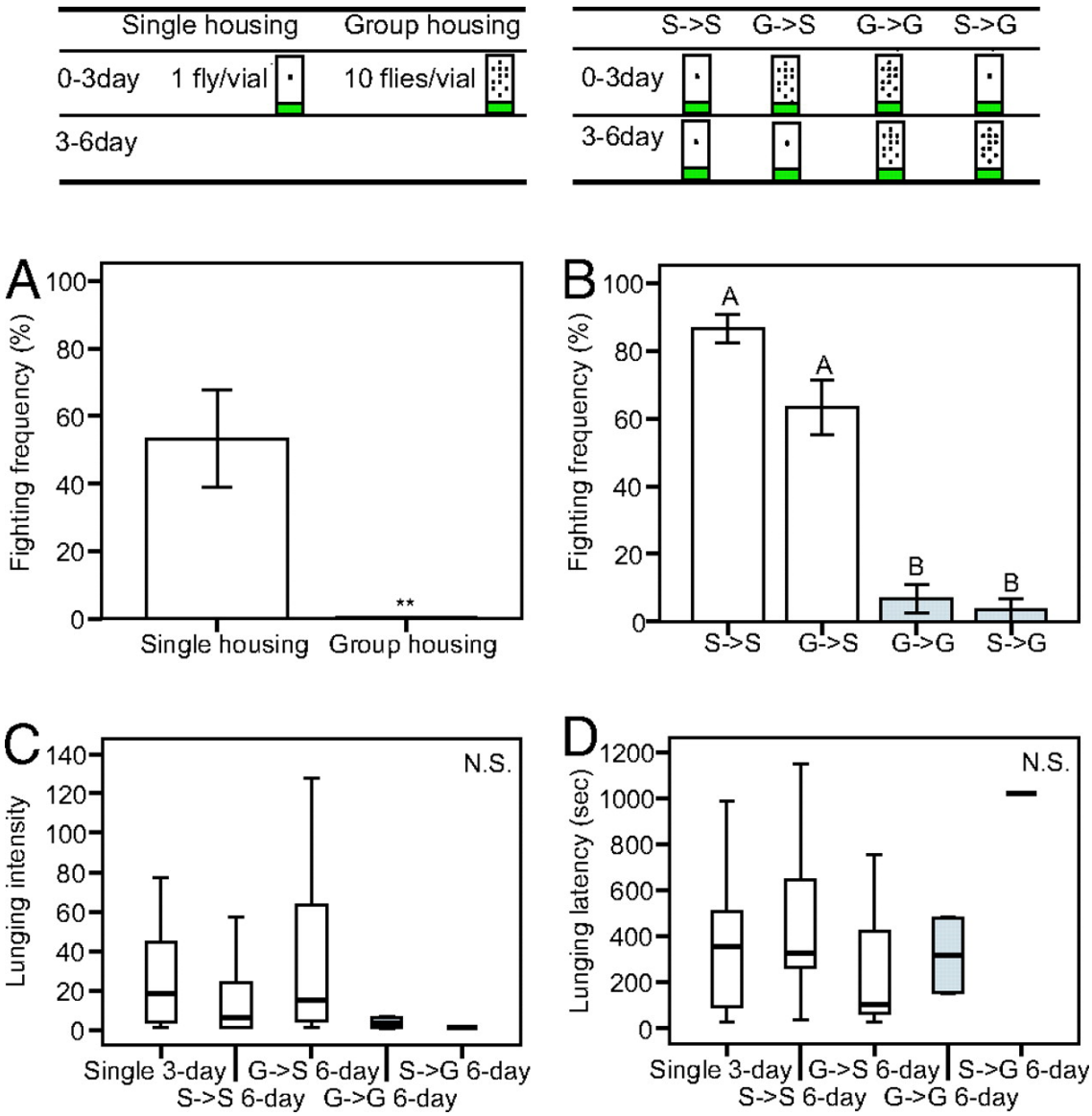
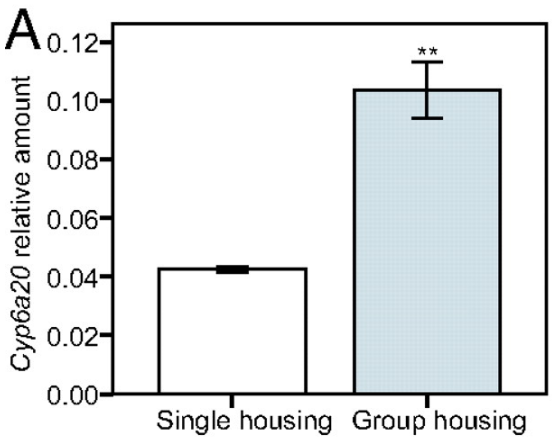
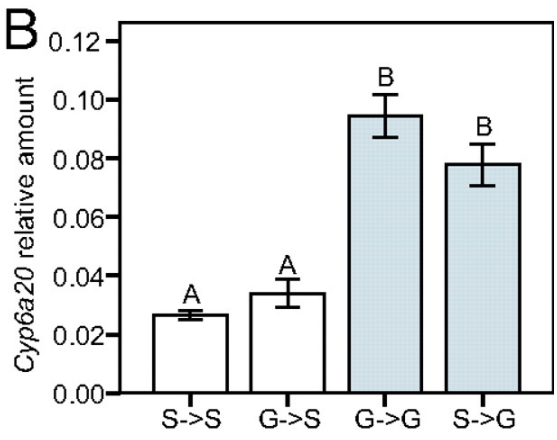

Single housing
Group housing
0-3day
1 fly/vial
10 flies/vial
3-6day
S->S
G->S
G->G
S->G
A
Fighting frequency (%)
100
80
60
40
20
0
**
Single housing
Group housing
B
Fighting frequency (%)
A
A
B
B
S->S
G->S
G->G
S->G
C
Lunging intensity
140
120
100
80
60
40
20
0
N.S.
Single 3-day
S->S 6-day
G->S 6-day
G->G 6-day
S->G 6-day
D
Lunging latency (sec)
1200
1000
800
600
400
200
0
N.S.
Single 3-day
S->S 6-day
G->S 6-day
G->G 6-day
S->G 6-day

Figure 2. *Cyp6a20* expression is correlated with social experience

Upper diagrams illustrate experimental manipulations. (A) Relative levels of *Cyp6a20* mRNA (normalized to *Ddc* mRNA levels) in 3-day-old, single-housed and group-housed flies (mean±s.e.m. n=4. **p<0.01). (B) Relative levels of *Cyp6a20* mRNA in 6-day-old, S→S, G→S, G→G and G→S flies (mean±s.e.m. n=4. Significant differences are indicated by letters above each bar.) (C) Negative correlation between relative levels of *Cyp6a20* mRNA and fighting frequency. The linear regression plot (R^2=0.922) is compiled using the data in Figs. 1A, B and 2A, B. Comparisons between groups were made using student's t test (A), or ANOVA followed by a *post hoc* test (B).


Single housing
Group housing
0-3day
1 fly/vial
10 flies/vial
3-6day
S->S
G->S
G->G
S->G
0-3day
3-6day


A
Cyp6a20 relative amount
0.12
0.10
0.08
0.06
0.04
0.02
0.00
**
Single housing
Group housing


B
Cyp6a20 relative amount
0.12
0.10
0.08
0.06
0.04
0.02
0.00
A
A
B
B
S->S
G->S
G->G
S->G

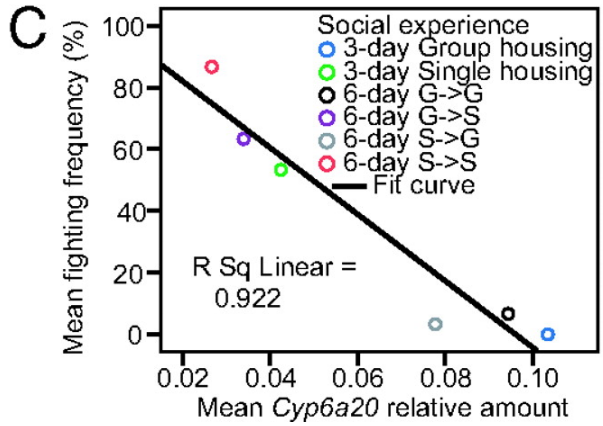

C
Mean fighting frequency (%)
100
80
60
40
20
0
Social experience
3-day Group housing
3-day Single housing
6-day G->G
6-day G->S
6-day S->G
6-day S->S
Fit curve
R Sq Linear =
0.922
0.02
0.04
0.06
0.08
0.10
Mean Cyp6a20 relative amount

Figure 3. *Cyp6a20* mutants exhibit increased aggressiveness only under group housing conditions

(A) Mean fighting frequencies of 6-day-old, single-housed and group-housed flies, of the indicated genotypes (mean±s.e.m. n=6 groups each containing 5 pairs; significant differences ($p<0.05$) are indicated by letters above the bars.) Comparison between groups was made using ANOVA followed by a *post hoc* test. (B) Median lunging intensities of 6-day-old, single- or group-housed flies of the indicated genotypes (n=9,11,10,2,15,1,17,13 pairs, respectively; N.S., $p>0.05$). (C) Median lunging latencies of 6-day-old, single- or group-housed flies of different genotypes (n=9,11,10,2,15,1,17,13 pairs, respectively; N.S., $p>0.05$). Comparisons between groups were made using Kruskal-Wallis ANOVA. (D) Two classes of negative genetic regulators of aggressiveness and their interaction with social experience. Class I genes, when mutated, increase aggressiveness under single-housing conditions, where their expression levels are normally relatively lower, but this phenotype can be overridden by group-housing. Class II genes, when mutated, increase aggressiveness under group- but not single-housing conditions, overriding the effect of social experience to suppress aggressiveness. *Cyp6a20* is a Class II gene.

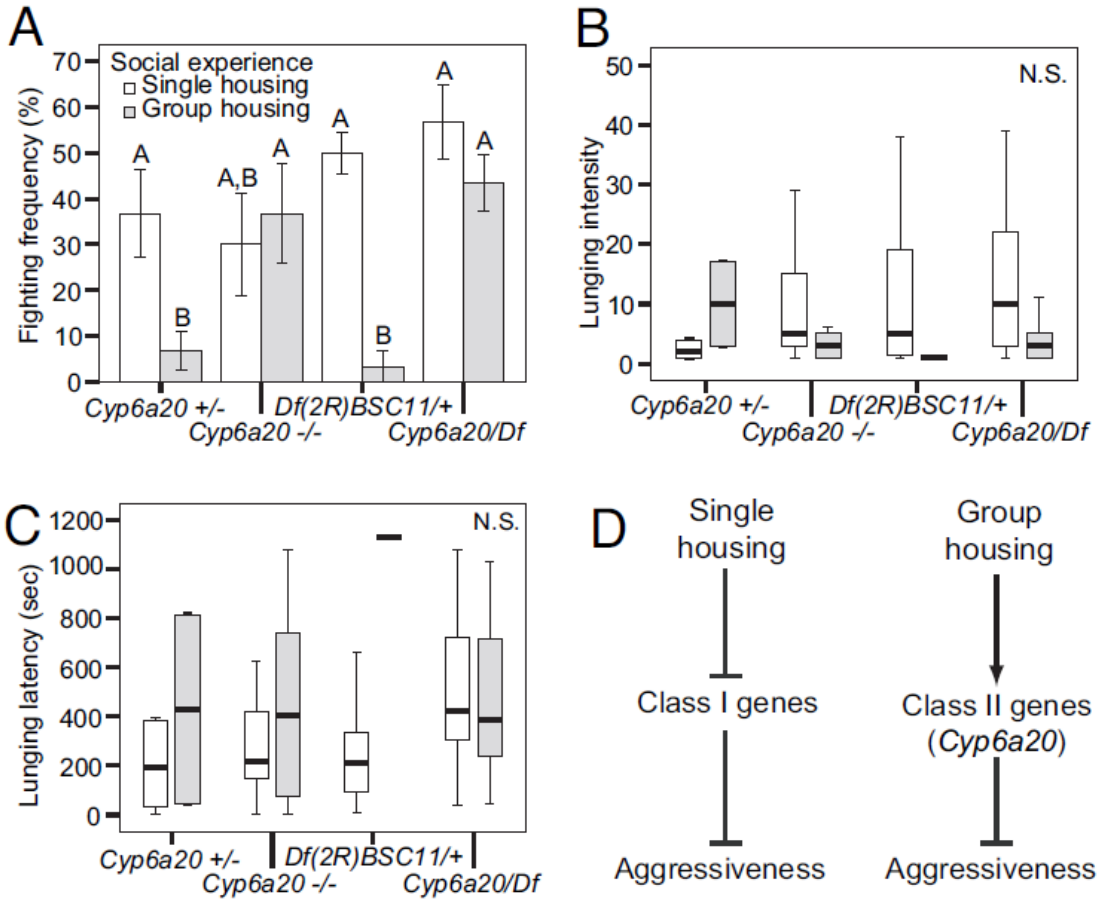

A
Social experience
Single housing
Group housing
Fighting frequency (%)
70
60
50
40
30
20
10
0
A
B
A,B
A
A
B
A
A
Cyp6a20 +/-
Cyp6a20 -/-
Df(2R)BSC11/+
Cyp6a20/Df
B
N.S.
Lunging intensity
50
40
30
20
10
0
Cyp6a20 +/-
Cyp6a20 -/-
Df(2R)BSC11/+
Cyp6a20/Df
C
N.S.
Lunging latency (sec)
1200
1000
800
600
400
200
0
Cyp6a20 +/-
Cyp6a20 -/-
Df(2R)BSC11/+
Cyp6a20/Df
D
Single housing
Class I genes
Aggressiveness
Group housing
Class II genes (Cyp6a20)
Aggressiveness

Figure 4. *Cyp6a20* mutants exhibit normal locomotor, olfactory and courtship behavior

(A) Mean walking distances of 6-day-old, single-housed and group-housed flies of the inidicated genotypes (n=20; N.S., p>0.05). Comparisons between groups were made using ANOVA followed by *post hoc* test. (B) Median number of flies of the indicated genotypes (n=15) trapped in food-containing (food+) vs. empty (food-) traps. Significant differences are indicated by letters above the bars). Comparisons between groups were made using Kruskal-Wallis ANOVA followed by a *post hoc* test. (C-F) *Cyp6a20* mutants have normal courtship behavior. (C) Percentage of fly pairs of the indicated genotypes that copulated in 30 minutes. (D) Mean courtship latency of flies of the indicated genotypes (n=21, 20, respectively; N.S., p>0.05). (E) Mean copulation latency of flies of the indicated genotypes (n=21, 20, respectively; N.S., p>0.05). (F) Mean courtship index of flies of the indicated genotypes (n=21, 20, respectively; N.S., p>0.05). For (D-F), comparisons between groups were made using Student's t test.

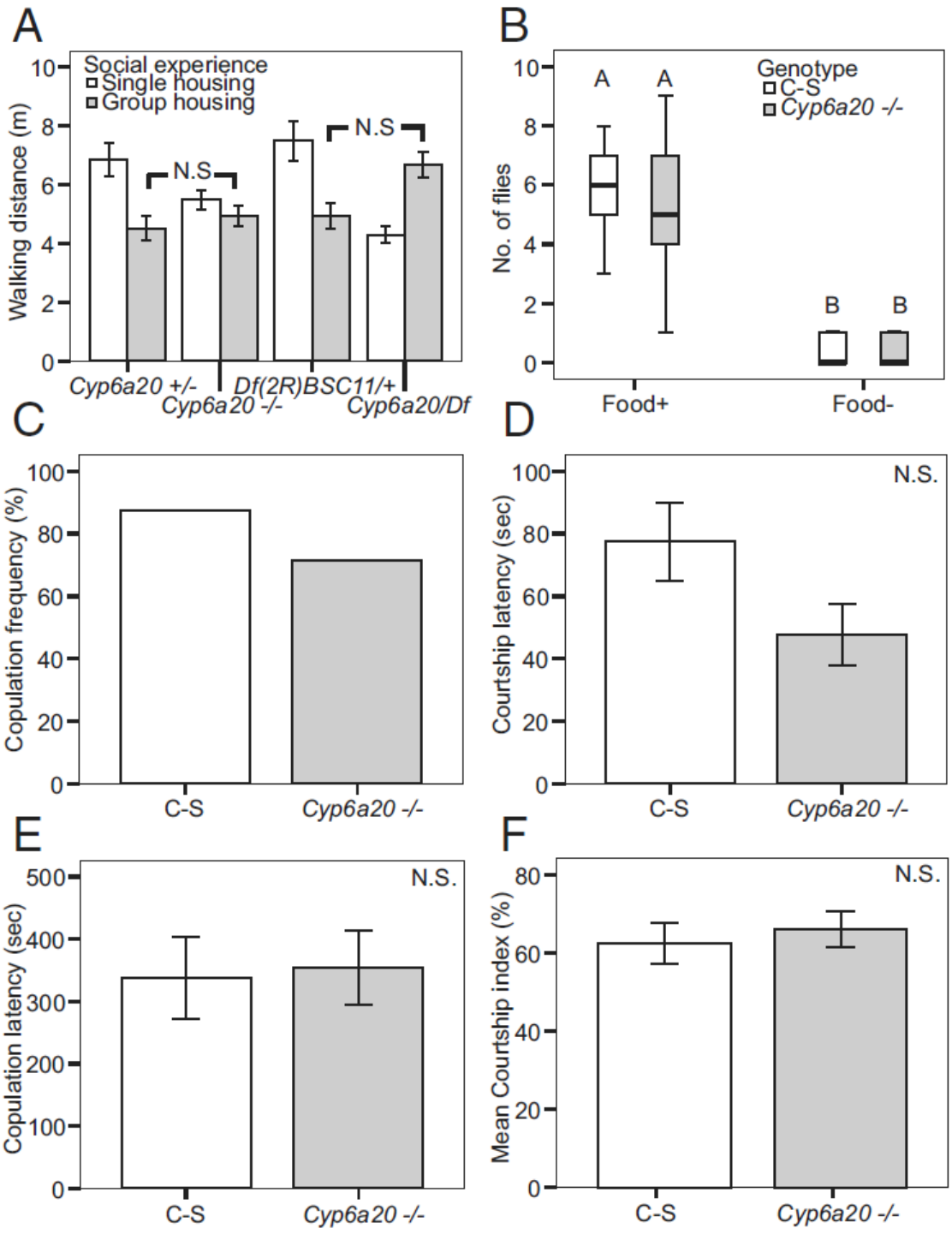

A
Social experience
Single housing
Group housing
Walking distance (m)
N.S
N.S
Cyp6a20 +/-
Cyp6a20 -/-
Df(2R)BSC11/+
Cyp6a20/Df
B
Genotype
C-S
Cyp6a20 -/-
No. of flies
A
A
B
B
Food+
Food-
C
Copulation frequency (%)
C-S
Cyp6a20 -/-
D
Courtship latency (sec)
N.S.
C-S
Cyp6a20 -/-
E
Copulation latency (sec)
N.S.
C-S
Cyp6a20 -/-
F
Mean Courtship index (%)
N.S.
C-S
Cyp6a20 -/-

Figure 5. *Cyp6a20* expression in olfactory sensory organs

(A, B) GFP expression in the antennae (A) and palps (B). Whole mount antennae and palps were stained with rabbit anti-GFP (green). (C, D) GFP^+ cells are non-neuronal. Frozen sections were stained with rabbit anti-GFP (green) and rat anti-ELAV (red). (E, F) a subset of GFP^+ cells in the antennae (E), (but not in the palps (F)), coexpressed LUSH. Frozen sections were stained with chick anti-GFP (green) and rabbit anti-LUSH (red). Confirmation of colabeling of one cell (white arrow) by z-series analysis is shown below and to the right of (E). Inset in (E) is a higher magnification view of the boxed region (arrowhead), illustrating a GFP^+, $LUSH^-$ cell and a GFP^+, $LUSH^+$ cell. In all images, TOPRO-3 (blue) was used for nuclear staining. (scale bars, 50 μm).

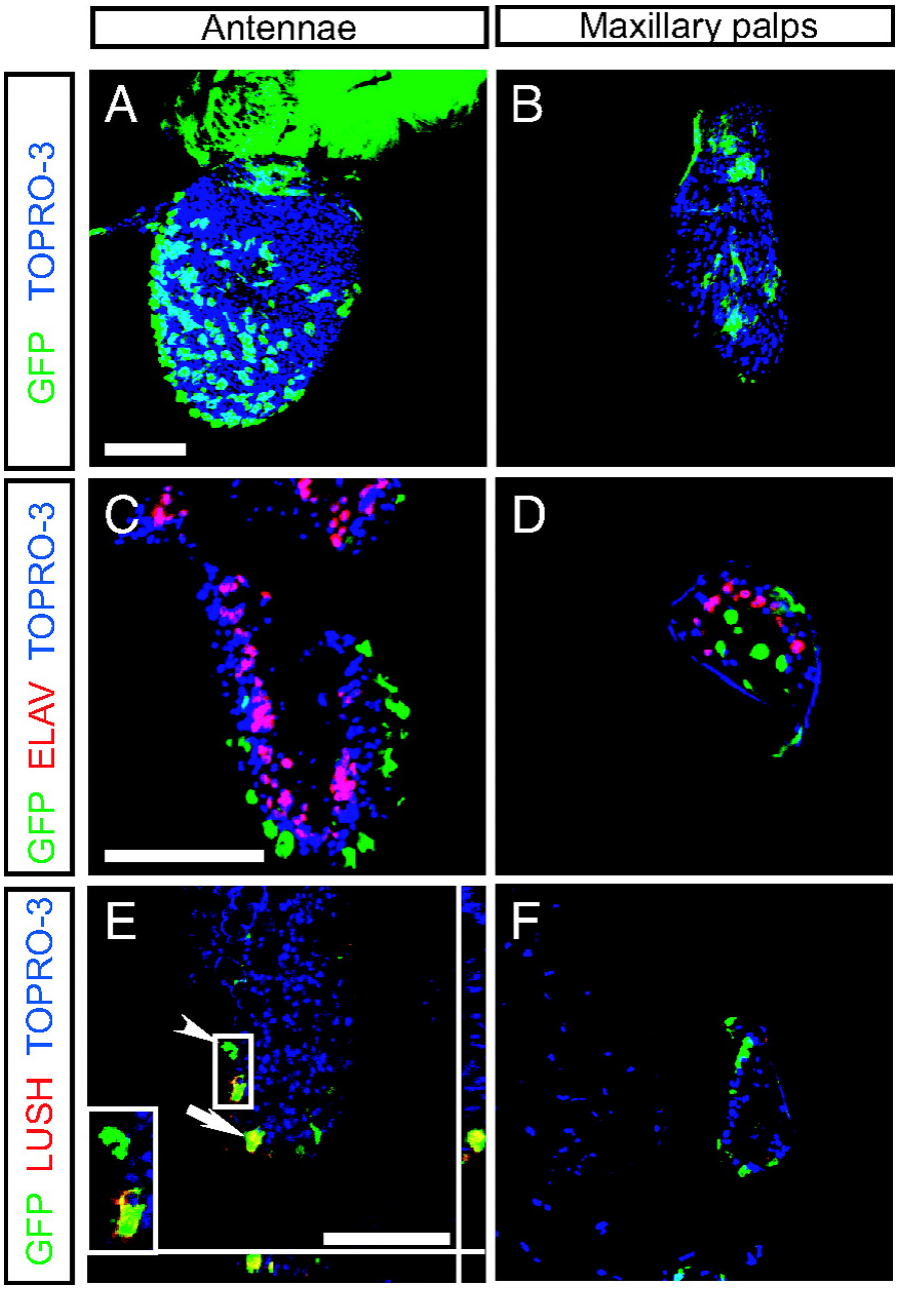
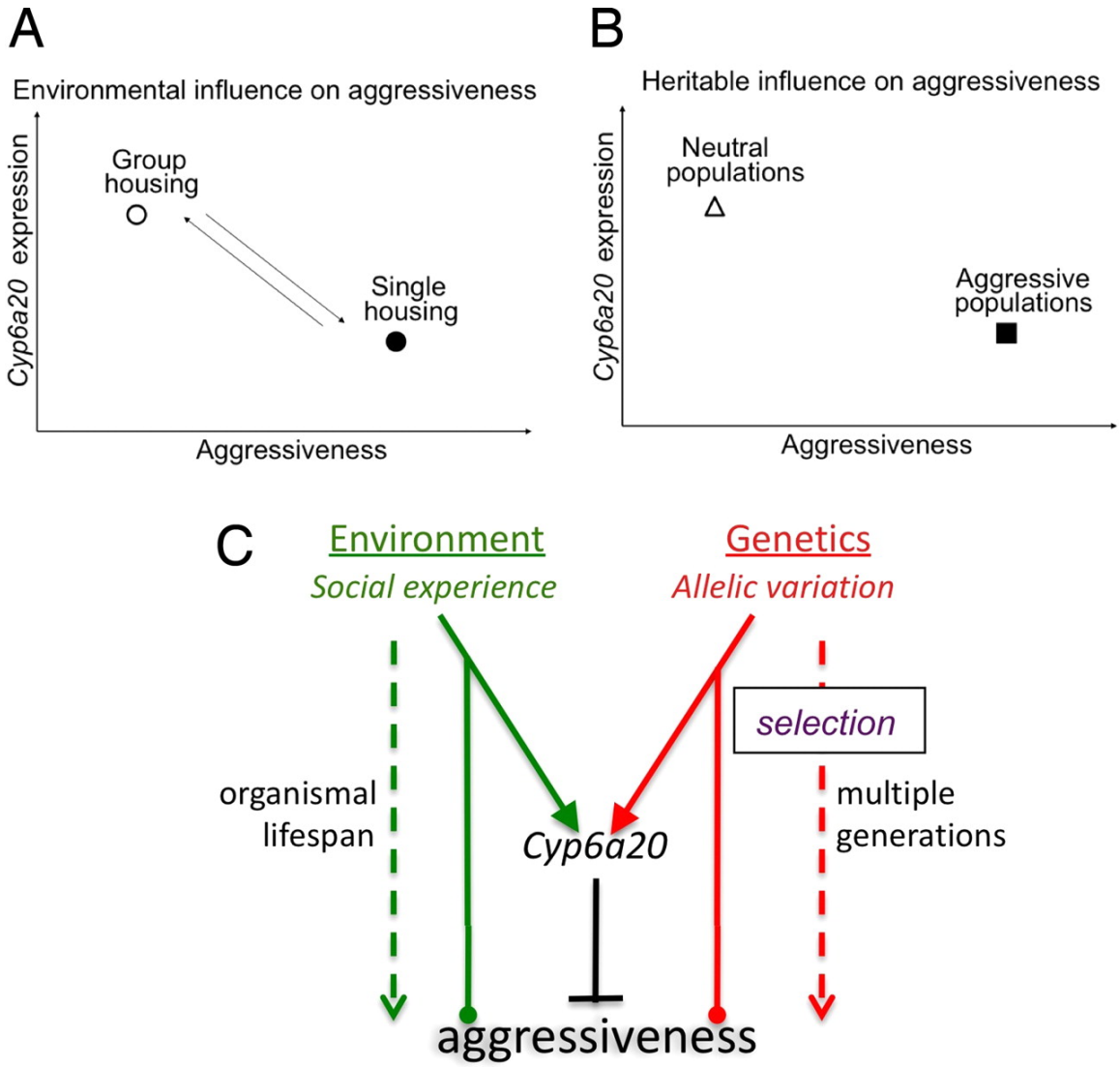

Antennae
Maxillary palps
A
B
C
D
E
F
GFP TOPRO-3
GFP ELAV TOPRO-3
GFP LUSH TOPRO-3

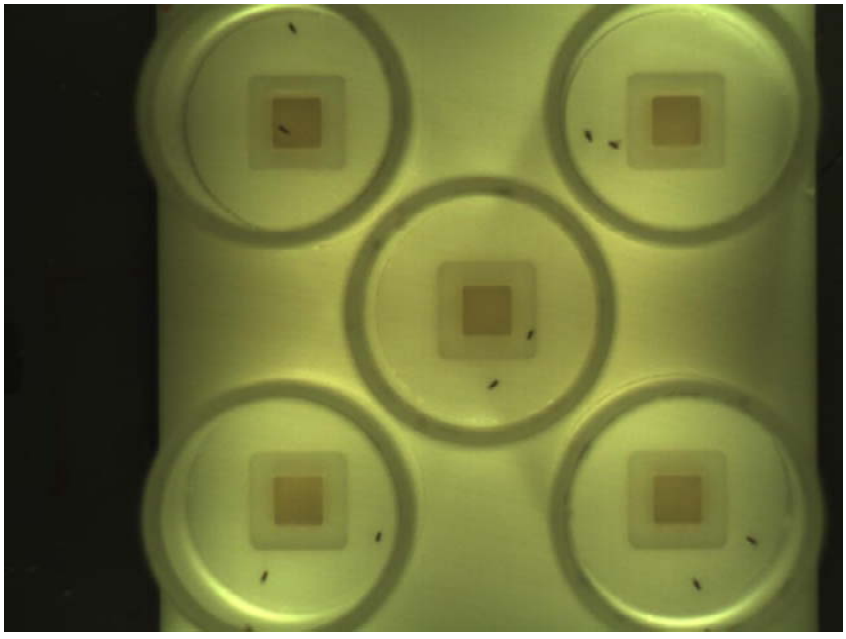
Figure 6. *Cyp6a20* is a common genetic target of environmental and heritable influences on aggressive behavior

(**A**) Social experience influences aggressiveness in a reversible manner (bi-directional arrows), mediated by differential expression of *Cyp6a20*. (**B**) Genetic selection over multiple generations establishes Neutral and Aggressive populations with differential levels of aggressiveness, which correlate with differential *Cyp6a20* expression. (C) Cyp6a20 regulation constitutes a common molecular target of environmental and genetic influences on aggressiveness. Circular arrowheads indicate that both positive and negative influences are possible. Environmental influences act on a timescale of the lifespan of the organism (left), while genetic influences act over multiple generations as a consequence of selection (natural or artificial).


A
Environmental influence on aggressiveness
Cyp6a20 expression
Group housing
Single housing
Aggressiveness
B
Heritable influence on aggressiveness
Cyp6a20 expression
Neutral populations
Aggressive populations
Aggressiveness
C
Environment
Social experience
Genetics
Allelic variation
selection
organismal lifespan
Cyp6a20
multiple generations
aggressiveness

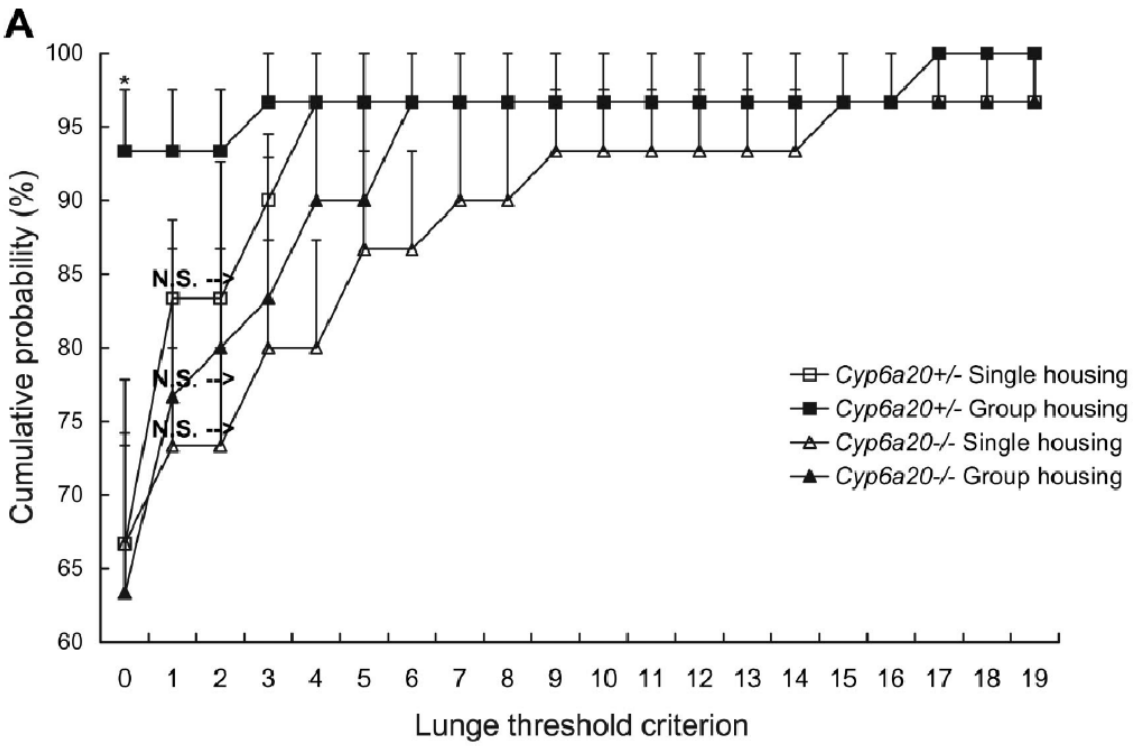
Supplementary Figure 1. Aggression arena

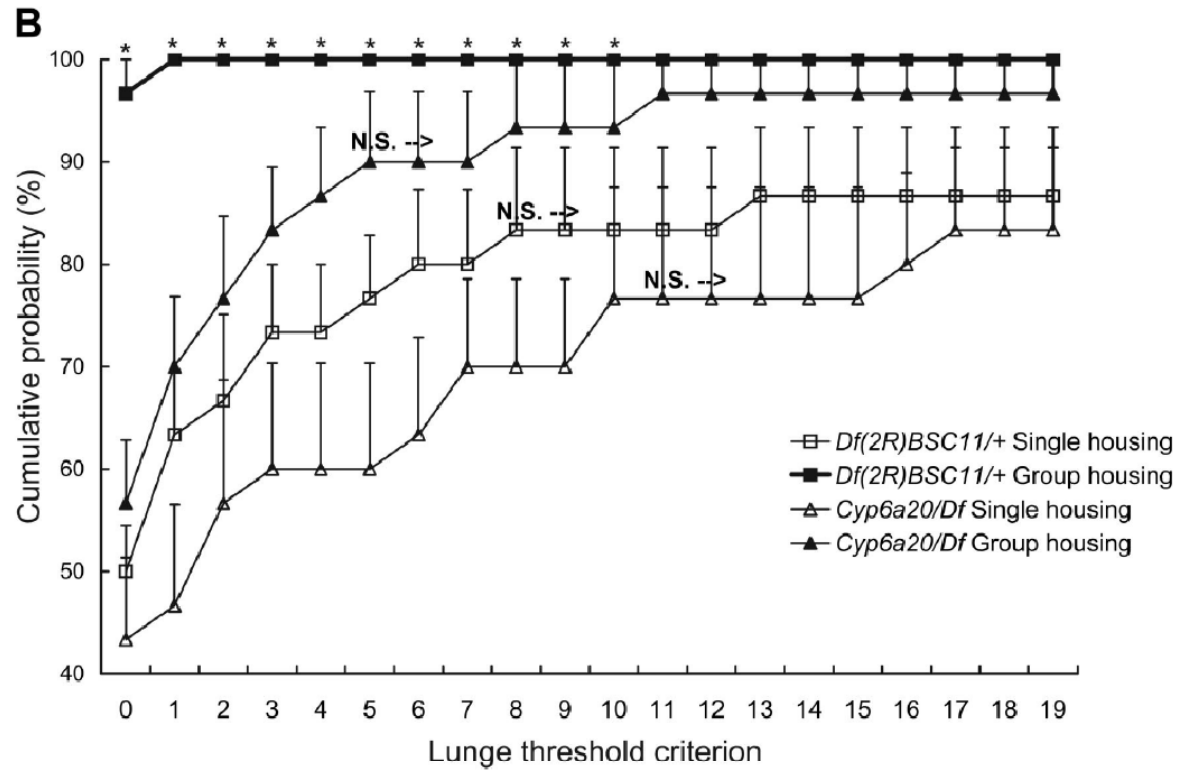
The apparatus was modified from an original design by Hoyer SC *et al.* [Hoyer SC, *et al.* (2008) Octopamine in male aggression of *Drosophila Curr Biol* 18:156–167]. The dimensions of each arena were 5.0 cm (*D*) X 11.4 cm (*H*). The center of the arena contains an inner square well (1.2 cm X 1.2 cm) filled with sucrose-apple juice-agar medium, and an outer square well (2.2 cm X 2.2 cm) filled with 1% agar medium. Fluon was painted on the inner surface of the wall of each arena, to prevent flies from climbing out. Arenas were covered by transparent plastic lids to prevent flies from escaping and to facilitate video capture.



Supplementary Figure 2. Cumulative probability distributions of lunging thresholds for *Cyp6a20* mutant analysis

For each lunge threshold criterion (*x* axis), the cumulative probability (*y* axis) represents the cumulative percentage of pairs that can be accounted for by *X* lunges or less (in 20 min). (*A*) Cumulative probability distributions for $Cyp6a20^{+/-}$ and $Cyp6a20^{-/-}$ (*n*=6, same datasets used in Fig. 3, error bars represent s.e.m.). (*B*) Cumulative probability distributions for $Cyp6a20^{Df/+}$ and $Cyp6a20^{Df/-}$ (*n*=6, same data used in Fig. 3, error bars represent s.e.m.). Curves that approach 100% more rapidly indicate a lower level of aggressiveness. Squares represent homozygous mutants ($Cyp6a20^{-/-}$ in *A* and *Cyp6a20*/*Df* in *B*), triangles represent heterozygous controls ($Cyp6a20^{+/-}$ in *A* and *Df(2R)BSC11*/+ in *B*). Open and solid symbols indicate single- and group-housing conditions, respectively (refer to Supplementary Text).

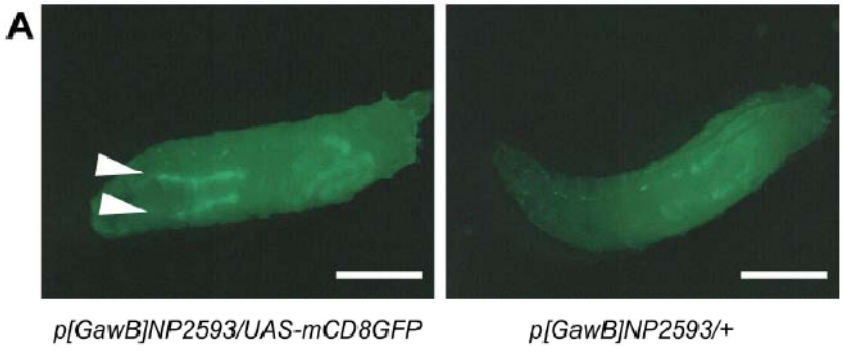

A
Cumulative probability (%)
100
95
90
85
80
75
70
65
60
*
N.S. -->
N.S. -->
N.S. -->
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Lunge threshold criterion
Cyp6a20+/- Single housing
Cyp6a20+/- Group housing
Cyp6a20-/- Single housing
Cyp6a20-/- Group housing

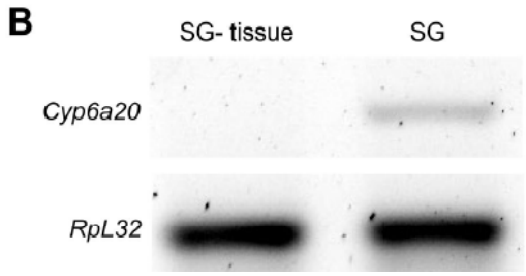

B
Cumulative probability (%)
100
90
80
70
60
50
40
* * * * * * * * * * * *
N.S. -->
N.S. -->
N.S. -->
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Lunge threshold criterion
Df(2R)BSC11/+ Single housing
Df(2R)BSC11/+ Group housing
Cyp6a20/Df Single housing
Cyp6a20/Df Group housing

Supplementary Figure 3. Larval *Cyp6a20* expression is enriched in salivary gland (SG)

For B, C-S third instar larvae were dissected in PBS and both SG and SG-removed larval tissue were collected. Equal amount of RNA extracted from both tissues were reverse-transcribed and simultaneously amplified using *RpL32* and *Cyp6a20* primers, producing 423 bp and 962 bp, respectively. *RpL32* (CG7939) was used as an internal control of gene expression. PCR products were loaded on 1.2% agarose gel and visualized by ethidium bromide.

(*A*) GFP expression was seen in SG of *P[GawB]NP2593*/*UAS-mCD8GFP* (*Left*, arrow head), but not in SG of *P[GawB]NP2593*/+ (*Right*) larva. (Scale bar: 1 mm.) (*B*) RT-PCR confirmed *Cyp6a20* transcripts were enriched in SG.





SUPPLEMENTARY TEXT

Cumulative probability distribution (CDP) analysis

The most sensitive criterion for fighting is that a given pair of flies exhibits at least one lunge during the 20 minute observation period. However, arbitrarily less sensitive (more stringent) criteria can also be used. We wished to examine systematically how criteria of different stringencies affected the comparison between housing conditions and genotypes. To do this, we first measured the total number of lunges exhibited by each pair of flies, during the 20 minute observation period. We then plotted the data as a cumulative probability distribution curve, for each genotype and housing condition (heterozygous/single-housed; homozygous/single-housed; heterozygous/group-housed; homozygous/group-housed). Due to different genetic backgrounds, separate comparisons were made for P-element heterozygotes vs. homozygotes (*Cyp6a20+/-* vs. *Cyp6a20-/-*, A), and Df heterozygotes vs. Df/P-element homozygotes (*Df(2R)BSC11/+* vs. *Cyp6a20/Df*, B).

In each case, group-housed heterozygous controls (A, *Cyp6a20+/-* and B, *Df(2R)BSC11/+*) were chosen as the "reference" datasets for comparison to other conditions/genotypes. For each stringency criterion (lunge number), a Kruskal-Wallis ANOVA (significance level=0.05) was performed on each dataset from the four curves. If there was significant difference between the four datasets, then a Mann Whitney U test (significance level=0.05) was performed between the "reference" dataset and the three other "test" datasets. This analysis permitted a comparison of the aggressiveness of different genotypes under different housing conditions, at progressively higher stringencies.

At the origin of each curve, when X=0, the group-housed heterozygous controls (A, *Cyp6a20+/-* Group housing, and B, *Df(2R)BSC11/+* Group housing) are significantly different from all other groups. As the lunge threshold criterion (X) increases, the difference between the four datasets becomes gradually indistinguishable. This is to be expected, because if the lunge threshold were set arbitrarily high (e.g., at least 1,000 lunges/20 min), then none of the pairs in any condition or genotype would be scored as having a fight (Fighting Frequency = 0), and therefore all the curves would be statistically indistinguishable. The question is, how do the different curves behave as they approach this point of statistical equivalence? The asterisks above the reference curve represent points that are statistically different from "test" datasets in some or all of the other three curves. "N.S." above the remaining curves represents the point after which these curves become indistinguishable from the reference curve.

Four major conclusions can be drawn from this analysis:

1) The behavior of the three "test" curves with respect to each other is clearly different in A and B. In general, flies containing the *Df(2R)BSC11* deficiency chromosome (B) are more aggressive than those containing the P-element insertion chromosome (A). This difference could reflect a difference in the genetic backgrounds of CS and *Df(2R)BSC11/Cyo* flies, or haplo-insufficiency of genes other than *Cyp6a20* that are encompassed by the deficiency. Therefore the most conservative conclusions are those that can be drawn from the analysis of the P-element insertion data (A).

2) The three "test" datasets in A and B are indistinguishable from each other by Kruskal-Wallis ANOVA, in the range X=0-19. In addition, all "test" datasets are

significantly different from the "reference" (heterozygous/group-housed) dataset for threshold criteria X=0 (A) or X=0-4 (B), at each of the individual lunge threshold values within these intervals. This indicates that the *Cyp6a20* homozygous mutation renders flies equally aggressive under group-housed and single-housed conditions, and that under both housing conditions, these mutant flies are more aggressive than group-housed heterozygous controls. This supports the conclusion that the suppressive effect of enriched social experience on aggressiveness is mediated, at least in part, by *Cyp6a20*.

3) The curves for homozygous P-element mutant flies, in either the single- or group-housing conditions, become statistically indistinguishable from the reference curve at the same lunge criterion (X=1, *Cyp6a20-/-* group housing, and *Cyp6a20-/-* single housing, A). This indicates that single-housed homozygous flies are no more aggressive than group-housed homozygous flies, in comparison to group-housed heterozygous flies. This would suggest that most or all of the difference in aggressiveness between single and group-housed heterozygous flies is due to *Cyp6a20,* and therefore that this gene is the primary, if not the exclusive, mediator of the effect of social experience on aggressiveness. However, in experiments using the deficiency-containing chromosome (B), the two homozygous curves reach statistical equivalence with the "reference" curve at different points (X=5 for *Cyp6a20/Df*, group housing, X=11 for *Cyp6a20/Df*, single housing, B). This indicates that under single housing conditions, the homozygous mutants are still somewhat more aggressive than they are under group housing conditions. Subject to the caveats in 1), this would imply that additional genes besides *Cyp6a20* mediate the effect of social experience on aggressiveness.

4) The curves for single-housed flies (homozygous or heterozygous) become indistinguishable from the reference curve at the same point in (A) (X=1, *Cyp6a20-/-,* single housing and *Cyp6a20+/-,* single housing). This implies that single-housed flies are no more aggressive in the presence or absence of *Cyp6a20*, in comparison to group-housed heterozygous flies. This would suggest that *Cyp6a20* exclusively functions in mediating the effect of group housing to suppress aggressiveness. However, in (B), the two single-housing curves reach statistical equivalence with the reference curve at different points (X=8 for *Df(2R)BSC11/+,* single housing, X=11 for *Cyp6a20/Df,* single housing). This suggests a difference of aggressiveness between single-housed heterozygous (*Df/+*) and homozygous (*Df/P-element*) *Cyp6a20* mutants. If so, then subject to the caveats in 1), *Cyp6a20* may function not only in mediating the effect of group housing on aggressiveness, but also in controlling the level of aggressiveness under single housing conditions.

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Chapter 3

Identification of an aggression-promoting pheromone and its receptor neurons in *Drosophila*

Liming Wang and David J. Anderson

SUMMARY

Aggression is regulated by pheromones in many animal species (Chamero et al., 2007; Keverne, 2002; Shorey, 1973). However in no system have aggression pheromones, their cognate receptors and corresponding sensory neurons been identified. Here we show that 11-*cis*-vaccenyl acetate (cVA), a male-specific volatile pheromone, robustly promotes male-male aggression in the vinegar fly *Drosophila melanogaster*. The aggression-promoting effect of synthetic cVA requires olfactory sensory neurons (OSNs) expressing the receptor Or67d (Ha and Smith, 2006; Kurtovic et al., 2007; van der Goes van Naters and Carlson, 2007), as well as the receptor itself. Activation of Or67d-expressing OSNs, either by genetic manipulation of their excitability or by exposure to male pheromones in the absence of other classes of OSNs, is sufficient to promote aggression. High densities of male flies can promote aggression through release of volatile cVA. In turn, cVA-promoted aggression can promote male fly dispersal from a food resource, in a manner dependent upon Or67d-expressing OSNs. These data suggest that cVA may mediate negative feedback control of male population density, through its effect on aggression. Identification of a pheromone-OSN pair controlling aggression in a genetic organism opens the way to unraveling the neurobiology of this evolutionarily conserved behavior.

Male-male aggression (hereafter referred to as "aggression") in the vinegar fly *Drosophila melanogaster* was first described almost a century ago (Sturtevant, 1915). Since then, considerable progress has been made in understanding its regulation (Baier et al., 2002; Chen et al., 2002; Dierick and Greenspan, 2006, 2007; Hoyer et al., 2008; Vrontou et al., 2006; Wang et al., 2008; Zhou et al., 2008). Nevertheless, little is known about how this behavior is controlled by sensory stimuli, in particular by pheromones. Recently, we showed that *Cyp6a20*, a cytochrome P450 gene previously identified by genetic selection for aggressiveness (Dierick and Greenspan, 2006), also mediates the influence of social experience on aggression(Wang et al., 2008). We found that *Cyp6a20* is expressed in pheromone-sensitive trichoid sensilla (van der Goes van Naters and Carlson, 2007) by support cells that co-express LUSH (Wang et al., 2008), an odorant binding protein required for detection of cVA (Laughlin et al., 2008; Xu et al., 2005), a male-specific volatile pheromone (Bartelt et al., 1985; Ejima et al., 2007; Kurtovic et al., 2007; Xu et al., 2005). These observations raised the question of whether cVA is involved in the pheromonal regulation of aggression in *Drosophila*.

We used CADABRA software (Dankert et al., 2009) to assess the influence of cVA on the behavioral interactions between pairs of Canton-S male flies. When 500 μg synthetic cVA was provided on a piece of filter paper in the behavior chamber (Hoyer et al., 2008) (Fig. 1g), a significantly higher number of lunges, the predominant aggressive behavior (Chen et al., 2002; Dankert et al., 2009; Hoyer et al., 2008), was observed (Fig. 1a). The effect of synthetic cVA to promote aggression was dose-dependent (Fig. 1m). Other aggressive behaviors, including wing threat (Fig. 1b), tussling (Fig. 1c) and chasing (Fig. 1d), were also up-regulated by addition of synthetic cVA (see ethograms in

Supplementary Fig. 1). The total walking distance of the fly pair was only modestly increased by cVA, and was unaltered if only a single fly was present (Fig. 1j), suggesting that the aggression-promoting effect of the pheromone is not due to an increase in locomotor activity (Hoyer et al., 2008).

Since courtship and aggression are opponent social behaviors that may reciprocally inhibit each other (Certel et al., 2007), we tested whether the stimulatory effect of synthetic cVA on aggression is associated with any influence on male-male courtship. No change in male-male courtship was observed in response to 500 μg of synthetic cVA, as measured by the occurrence of unilateral wing extension (Fig. 1e), or circling behavior (Fig. 1f). Thus cVA promotes aggression without altering the frequency of male-male courtship behaviors (Fig. 1h,i, and Supplementary Fig. 1).

cVA has also been shown to suppress male mating behavior towards females (Ejima et al., 2007; Kurtovic et al., 2007). However, under our conditions 500 μg of synthetic cVA was insufficient to suppress such behavior, as measured by cumulative latency to copulation (Fig. 1k). The effect of cVA to promote aggression can, therefore, be observed under conditions where the pheromone does not affect male sexual behaviors. Nevertheless, 5 mg of synthetic cVA was sufficient to suppress male-female mating (Fig. 1l), while no further increase in lunging was observed using this higher amount of cVA (Fig. 1m). Thus, synthetic cVA can regulate two different male social behaviors, aggression and mating, in opposite directions with different dosage requirements.

Two different olfactory receptors, Or67d and Or65a, have been identified as cVA receptors (Ejima et al., 2007; Ha and Smith, 2006; Kurtovic et al., 2007; van der Goes van Naters and Carlson, 2007). Silencing Or67d-expressing OSNs by expressing the inwardly rectifying potassium channel Kir2.1 (Baines et al., 2001) blocked the effect of synthetic cVA to promote aggression (Fig. 2a). This effect of cVA was also eliminated in $Or67d^{GAL4/GAL4}$ mutant flies (Kurtovic et al., 2007) (Supplementary Fig. 2a), indicating that Or67d receptors, as well as Or67d-expressing OSNs, are required. Consistent with a previous report that the *Or67d* gene is required for the mating-suppressing effect of synthetic cVA (Kurtovic et al., 2007), silencing Or67d-expressing OSNs blocked the effect of cVA to suppress male mating towards females (Fig. 2b). In contrast, silencing Or65a-expressing OSNs did not impair either promotion of aggression or suppression of male-female mating by cVA (Fig. 2d,e). These data suggest that synthetic cVA exerts its aggression-promoting effect, as well as its mating-suppressing effect, via Or67d-expressing OSNs.

We then asked whether increasing the excitability of Or67d-expressing OSNs is sufficient to promote aggression, in the absence of added cVA, by expressing a bacterially-derived sodium channel ("NaChBac") (Ren et al., 2001) in Or67d-expressing OSNs. Pairs of $Or67d^{GAL4}$*/UAS-NaChBac* male flies exhibited a significantly increased number of lunges in comparison to $Or67d^{+}$*/UAS-NaChBac* controls (Fig. 2c). These data indicate that increasing the excitability of Or67d-expressing OSNs can enhance aggression, and that the magnitude of this effect is similar to that obtained by addition of synthetic cVA. Activation of Or65a-expressing OSNs, in contrast, did not promote aggression (Fig. 2f). Negative results obtained using the *Or65a-GAL4* driver should be

interpreted with caution, however, because its strength may not be equivalent to that of *Or67d*^{GAL4} driver (Ejima et al., 2007).

As endogenously produced cVA is able to activate Or67d-expressing OSNs (Laughlin et al., 2008), we next asked whether these neurons are sufficient to mediate the effect of endogenously produced cVA to promote aggression. Male flies bearing a null mutation in *Or83b,* which encodes an obligatory co-receptor for olfactory receptors expressed in ~70-80% of OSNs (Larsson et al., 2004), exhibited a significant reduction in lunging behavior (Fig. 3a). This aggression deficit could be rescued by expressing an *Or83b* cDNA under the control of an *Or83b-GAL4* driver (Fig. 3b). These data indicate an essential role for one or more classes of Or83b-expressing OSNs, and thereby implicate one or more volatile pheromones released by male flies, in aggression.

We then tested whether restoring *Or83b* expression selectively in Or67d-expressing OSNs could also rescue the aggression deficit of *Or83b*$^{-/-}$ mutant males. Indeed, this manipulation produced a significant rescue of the reduced aggression phenotype of *Or83b*$^{-/-}$ mutants, to a level ~80% of that obtained using the *O83b-GAL4* driver (Fig. 3c). *Or83b*$^{-/-}$ mutant males expressing *Or83b* under the control of *Or65a-GAL4,* in contrast, did not exhibit a significant rescue of the aggression phenotype, although there was a slight trend in this direction (Fig. 3d). As Or67d-expressing OSNs respond essentially exclusively to cVA (Schlief and Wilson, 2007), these results indicate that activation of Or67d OSNs by endogenously produced cVA is sufficient to promote aggression, when all other classes of Or83b-expressing OSNs are inactive. Taken together, these gain-of-function data indicate that activation of Or67d-expressing OSNs

(using NaChBac) is sufficient to promote aggression, and that these neurons are sufficient to mediate the aggression-promoting effect of endogenous cVA.

As cVA is a volatile pheromone, its concentration should be proportional to the number of male flies in a given environment. If so, then increased levels of aggression might be observed in a setting containing a high density of male flies, in a cVA-dependent manner. To eliminate the confound that a higher density of male flies could produce a higher number of lunges per fly pair simply because of an increased frequency of interaction, we developed an assay to examine the effect of a high density of "caged" male flies on the aggressiveness of a single pair of neighboring "tester" males. The "donor" caged male flies were separated by a meshed compartment from the "tester" males, permitting transmission of volatile odorants while preventing physical interactions between the "donor" and "tester" males (Fig. 4a). The "tester" males indeed performed a higher number of lunges in the presence of "donor" males, in a manner proportional to the number of these caged donors (Fig. 4b). Importantly, the ability of the "donor" males to enhance aggression was eliminated by silencing Or67d-expressing OSNs in the "tester" males (Fig. 4c), or by eliminating the *Or67d* gene (Supplementary Fig. 2b). These data indicate that proximity to a high density of male flies can increase the level of aggression, and that this increase is mediated predominantly, if not exclusively, by release and detection of endogenous cVA.

The observation that aggression is enhanced by proximity to a high density of male flies raised the question of whether aggressive behavior might, in turn, regulate population density. Aggressive males chase competing males (Chen et al., 2002) from a

resource as part of their territorial behavior (Hoffmann, 1987), and thereby disperse them. If a high fly density promotes aggression via elevated levels of cVA, and if increased aggression in turn enhances dispersal, then cVA-promoted aggression might ultimately limit the density of male flies on a given resource. To test this hypothesis, we first examined whether synthetic cVA promotes fly dispersal in a setting where multiple (six) male flies compete for a limited food resource territory (Fig. 4d). In the absence of synthetic cVA, control *Or67d*$^{+}$*/UAS-Kir2.1* and *Or67d*^{GAL4}/+ male flies quickly congregated on the food resource and remained there for at least 30 minutes after introduction into the chamber (Fig. 4e,f, blue line). In the presence of synthetic cVA, however, the number of these control male flies on the food cup declined following their initial attraction to the resource, indicating dispersal (Fig. 4e,f, green line). cVA did not promote dispersal from the food resource if individual male flies, instead of 6 flies, were introduced into the behavior chamber (data not shown), suggesting that the dispersal observed in the 6-fly assays is due to aggression. Indeed, under these conditions cVA also robustly promoted aggression (Supplementary Fig. 3a). In contrast, *Or67d*^{GAL4}*/UAS-Kir2.1* male flies exhibited neither increased dispersal (Fig. 4g), nor increased aggression (Supplementary Fig. 3a), in response to synthetic cVA. These data suggest that cVA promotes dispersal of male flies through Or67d-expressing OSNs. Consistent with this interpretation, increasing the excitability of Or67d-expressing OSNs (using "NaChBac") promoted dispersal and aggression in a manner similar to that of exogenously added cVA (Fig. 4h and Supplementary Fig. 3b).

These data indicate that activation of cVA-responsive OSNs can reduce the number of male flies on a food resource, by promoting aggression. cVA has also been

reported to function as an aggregation pheromone in *Drosophila* (Bartelt et al., 1985; Xu et al., 2005). Taken together, these data suggest that cVA may play a role in the homeostatic control of male fly population density on a food resource: at low population densities, cVA causes more flies to accumulate on the resource via its aggregation-promoting function; once the population density of male flies increases above some threshold, the increased levels of cVA promote aggression and dispersal, thereby reducing the population density to a level that achieves an optimal balance between feeding, reproduction and competition (Fig. 4i). How the different behavioral functions of cVA are exerted through a common population of OSNs is an interesting question for future study (Benton, 2007).

The control of aggression by pheromones and other semiochemicals in insects has been extensively studied (Kou et al., 2006; Ono et al., 2003; Shorey, 1973). Such studies have established correlations between the quantity of certain pheromones in tissue extracts and the intensity of aggressive behavior (Kou et al., 2006), and in some cases have demonstrated the ability of such pheromones, in pure or synthetic form, to promote aggression (Ono et al., 2003). However, with few exceptions (Chamero et al., 2007), it has been difficult to establish whether these pheromones actually play a physiological role in regulating aggression. The genetic tools available in *Drosophila* have permitted us to establish that cVA, both in synthetic form and when released endogenously by male flies, promotes aggression in this species, via Or67d-expressing OSNs and the Or67d receptor itself. Further dissection of the circuits engaged by these OSNs (Datta et al., 2008) should facilitate our understanding of the neurobiology of this evolutionarily conserved, innate social behavior.

ACKNOWLEDGEMENTS

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METHODS

Behavioral assays

Behavioral assays in most experiments were performed using 5-6 day-old male flies that were raised after eclosion in groups of 10 flies/vial prior to testing. Single-housed flies were used in the experiments shown in Fig. 3, in order to provide a higher level of baseline aggression against which to evaluate decreases in aggression caused by the *Or83b* mutations. In all experiments involving genetic manipulations, comparisons between genotypes were made on equivalent genetic backgrounds. $Or67d^{GAL4/GAL4}$ flies contain an insertion of *GAL4* into the chromosomal *Or67d* gene that produces a null mutation; the genetic control for this allele, $Or67d^{+/+}$, is derived by excision of the *GAL4* insertion, reverting *Or67d* to a functional gene[6].

Most experiments (Fig. 1-3) were performed in a behavior chamber similar to that described previously[14] except that the floor was uniformly filled with apple juice-sugar-agar medium (Fig. 1a). Two males (taken from different housing vials) were introduced into the chamber by gentle aspiration without anesthesia, videotaped for 20 minutes and behavioral data extracted from the videotape using CADABRA (Dankert et al., 2009) software. For mating assays between males and virgin females, the latency to copulation was scored manually. Where indicated, synthetic cVA dissolved in acetone (or acetone alone as a control) was delivered by spotting onto a small piece of filter paper placed in one corner of the arena.

Different chamber designs were used for the experiments involving caged "donor" males (Fig. 4a), or males competing for a food resource (Fig. 4d), and the number of lunges (Fig. 4b-c) or the number of flies on the food cup (Fig. 4e-h) were scored manually for these experiments. Detailed descriptions of fly stocks, experimental designs and statistical analysis are provided in the Supplementary Methods.

Fly stocks and rearing conditions

All fly stocks were reared on medium containing yeast, corn syrup, and agar at 25°C and 60% humidity, on a 12-h light:12-h dark cycle. Newly eclosed males were reared either individually (single-housed) or at 10 flies (group-housed) per vial [2.4cm (Diameter) x 9.4cm (Height)] for 5-6 days before the behavioral assays. Virgin females were also collected shortly after eclosion and reared at 20 females per vial for 5-6 days before courtship assays. Canton-S flies were obtained from M. Heisenberg; $Or67d^{+/+}$ and $Or67d^{GAL4/GAL4}$ flies were from B. Dickson; *Or67d-GAL4*, *Or65a-GAL4*, *Or83b-GAL4*, *UAS-Or83b* and $Or83b^{-/-}$ flies were from L. Vosshall.

Experimental design

Experiments shown in Fig. 1-3 were performed in the behavior chamber shown in Fig. 1g. The chamber was coated with Fluon and placed on top of a thin layer of apple juice-sugar-agar medium. The arena was illuminated by a fluorescent light bulb underneath. In some experiments, acetone (solvent) or acetone containing a given amount of liquid cVA (Phenobank) was applied to a small piece of filter paper and placed in one corner of the arena. Two flies were introduced into the arena by gentle aspiration and

their activities videotaped for 20 minutes and analyzed using CADABRA (Dankert et al., 2009) software. For mating assays between males and virgin females, the latency to copulation was scored manually. Cumulative copulation latency curves represent the percentage of male-female pairs that copulate as a function of time.

For the experiments shown in Fig. 4b-c, a plastic tube coated with Fluon was used as the behavior chamber as shown (Fig. 4a). A 70 μm cell strainer (BD Falcon) was placed in the center as the meshed compartment. The indicated number of male or female "donor" flies was transferred into this compartment using light anesthesia at least one hour before the behavioral assays. A drop of yeast paste was placed onto the center of the meshed compartment. A pair of group-housed "tester" males of the indicated genotype was introduced into the chamber, but outside the meshed compartment, by gentle aspiration and videotaped for 20 minutes. The total number of lunges performed on the meshed compartment was scored manually.

For the experiments shown in Fig. 4e-h, a plastic tube coated with Fluon was used as the behavior chamber and a small cup containing standard fly food was place in the center (Fig. 4d). In experiments shown in Fig. 4e-g, acetone or acetone containing 500 μg cVA was applied to a small piece of filter paper and placed on the food cup. 6 group-housed male flies of the indicated genotype were introduced into the chamber simultaneously and videotaped for 30 minutes. The total number of lunges was scored manually and the total number of flies on top of the food cup was counted every 30 seconds.

Statistical analysis

Mann-Whitney U test (for pair-wise comparisons) and Kruskal-Wallis ANOVA (for comparisons among >2 groups) were applied unless otherwise noted. Significant difference among groups detected by Kruskal-Wallis ANOVA were analyzed using Dunn's post-test (including a correction for multiple comparisons) as the *post hoc* test to identify groups exhibiting statistically significant differences. For the comparisons between cumulative copulation latencies, 2-way ANOVA was applied. For Fig. 4e-h, two-way ANOVA was used to identify data points that show significant differences ($p<0.05$).

FIGURES

Figure 1. Synthetic cVA promotes aggression

(a-f) Number of (a) lunges, (b) wing threats, (c) tussles, (d) chases, (e) unilateral wing extensions and (f) circling behaviors (per 20 minutes) performed by pairs of Canton-S (CS) males in the presence of solvent alone or 500 μg cVA (n=28-30). The temporal distribution of behaviors is shown in raster plots to the right of each panel. Each row of spikes represents one fly pair, and each spike represents one occurrence of the behavior. The histogram integrates the occurrences of each behavior in 1-minute bins. (g) Illustration (to scale) of the behavior chamber used for experiments shown in Fig. 1-3. (h,i) Number of all aggressive (h) and courtship (i) actions, based on the data in (a-f). (j) Walking distance of pairs of (n=28-30) or individual (n=16) CS males in the presence of solvent alone or 500 μg cVA. (k,l) Cumulative latency of CS males to copulate with virgin CS females in the presence of solvent (acetone) alone or 500 μg cVA (k; n=29), or solvent alone or 5 mg cVA (l; n=30). (m) Number of lunges performed by pairs of C-S males in the presence of solvent alone, 100 μg cVA, 500 μg cVA or 5 mg cVA (n=20-24). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. Error Bars are s.e.m. in this and all figures.

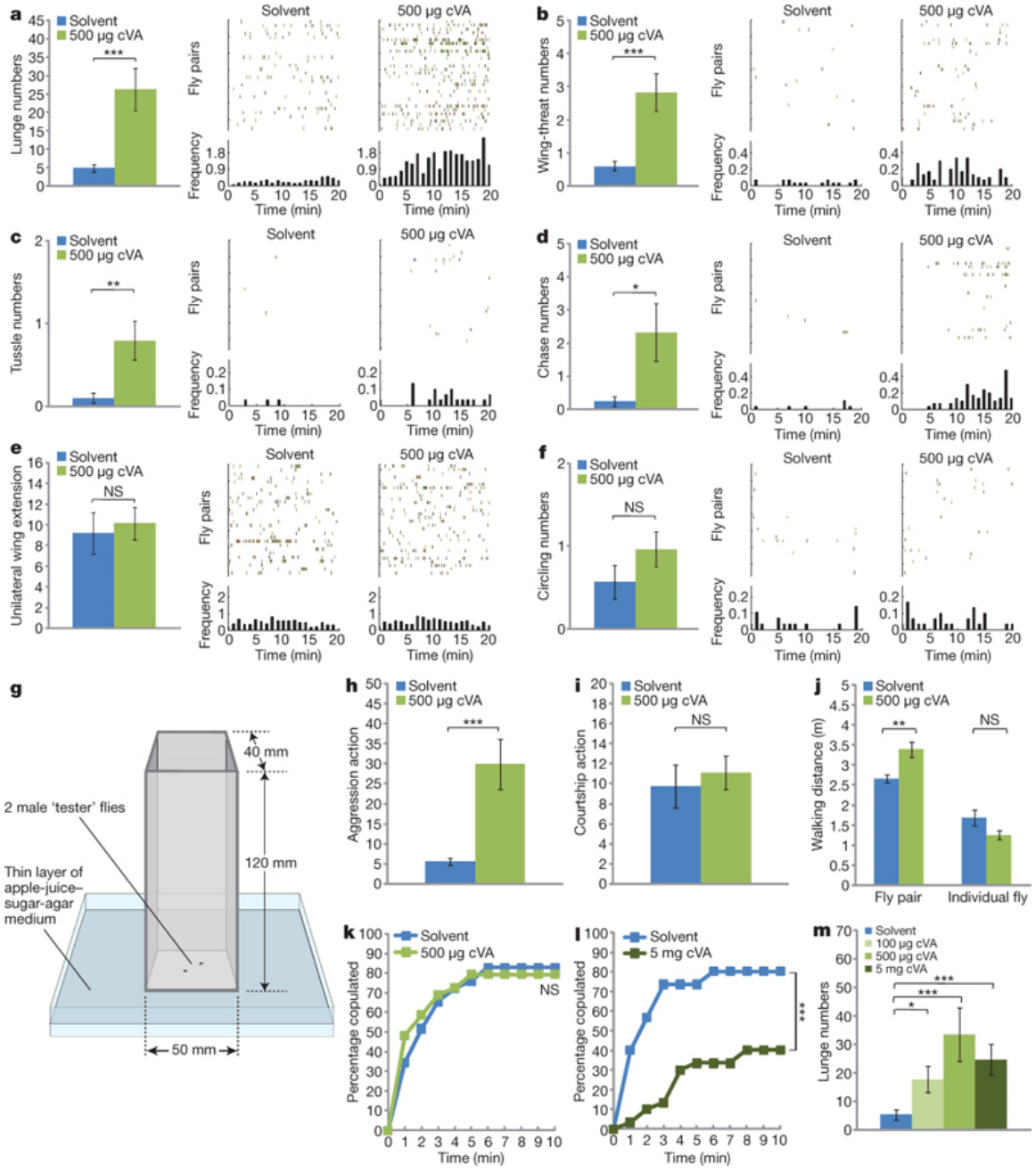

a
Solvent
500 μg cVA
***
Lunge numbers
Fly pairs
Frequency
Time (min)
b
Wing-threat numbers
c
Tussle numbers
**
d
Chase numbers
*
e
Unilateral wing extension
NS
f
Circling numbers
g
2 male 'tester' flies
Thin layer of apple-juice–sugar-agar medium
40 mm
120 mm
50 mm
h
Aggression action
i
Courtship action
j
Walking distance (m)
Fly pair
Individual fly
k
Percentage copulated
l
5 mg cVA
m
100 μg cVA

Figure 2. Or67d-expressing OSNs mediate the aggression-promoting effect of synthetic cVA.

(a,c,d,f) Number of lunges (per 20 minutes) performed by pairs of males of the indicated genotype, in the presence of solvent alone or 500 μg cVA (a,d; n=18-20), or with no added pheromone (c,f; n=20-26). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. (b,e) Cumulative latency of males of the indicated genotype to copulate with virgin females in the presence of solvent alone or 5 mg cVA (n=20-28). Note that silencing of Or67d-expressing OSNs impairs the suppression of mating by cVA (green open circles in b). *** $P<0.001$.

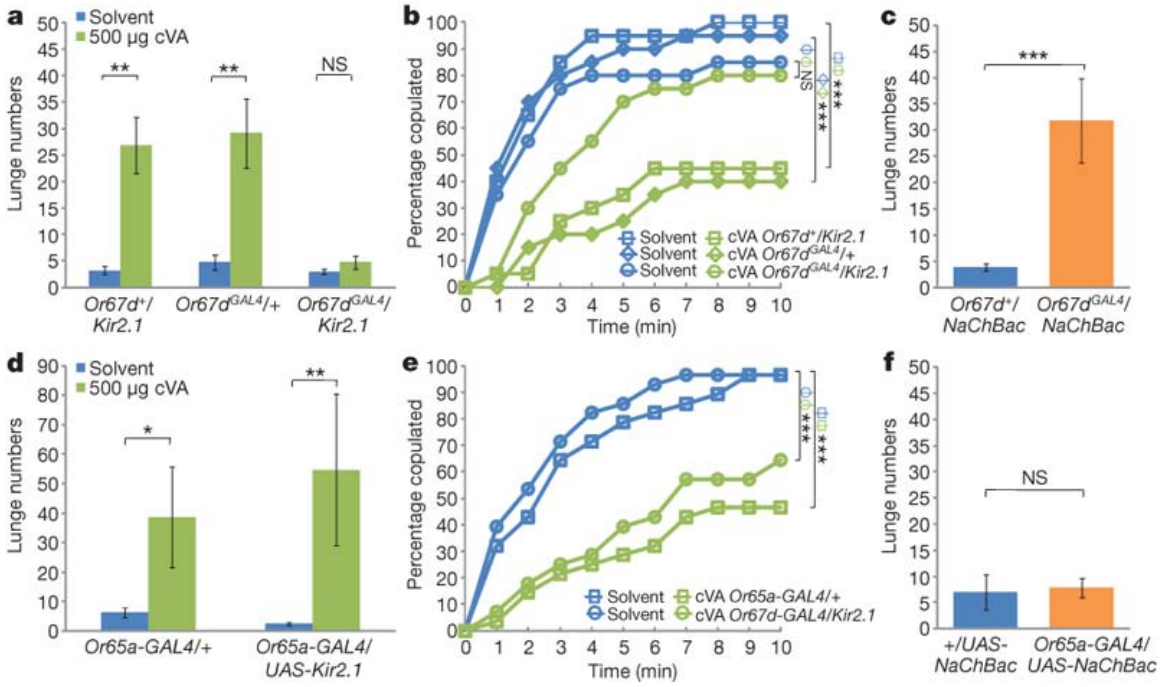

a
Solvent
500 µg cVA
Lunge numbers
**
**
NS
Or67d+/ Kir2.1
Or67dGAL4/+
Or67dGAL4/ Kir2.1
b
Percentage copulated
Time (min)
Solvent
cVA Or67d+/Kir2.1
Solvent
cVA Or67dGAL4/+
Solvent
cVA Or67dGAL4/Kir2.1
NS
****
****
c
Lunge numbers
***
Or67d+/ NaChBac
Or67dGAL4/ NaChBac
d
Solvent
500 µg cVA
Lunge numbers
*
**
Or65a-GAL4/+
Or65a-GAL4/ UAS-Kir2.1
e
Percentage copulated
Time (min)
Solvent
cVA Or65a-GAL4/+
Solvent
cVA Or67d-GAL4/Kir2.1
****
****
f
Lunge numbers
NS
+/UAS-NaChBac
Or65a-GAL4/ UAS-NaChBac

Figure 3. Or67d-expressing OSNs are sufficient to mediate the aggression-promoting effect of endogenously produced cVA.

In all graphs, the number of lunges (per 20 minutes) performed by pairs of males of the indicated genotype is shown (n=20-34). For this experiment, single-housed male flies, which exhibit a higher baseline level of aggression(Wang et al., 2008) were used in order to more readily detect the decreased aggression caused by the *Or83b* mutation. Note that restoration of *Or83b* expression to Or67d-expressing neurons rescues the loss of aggressiveness in $Or83b^{-/-}$ flies (c). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

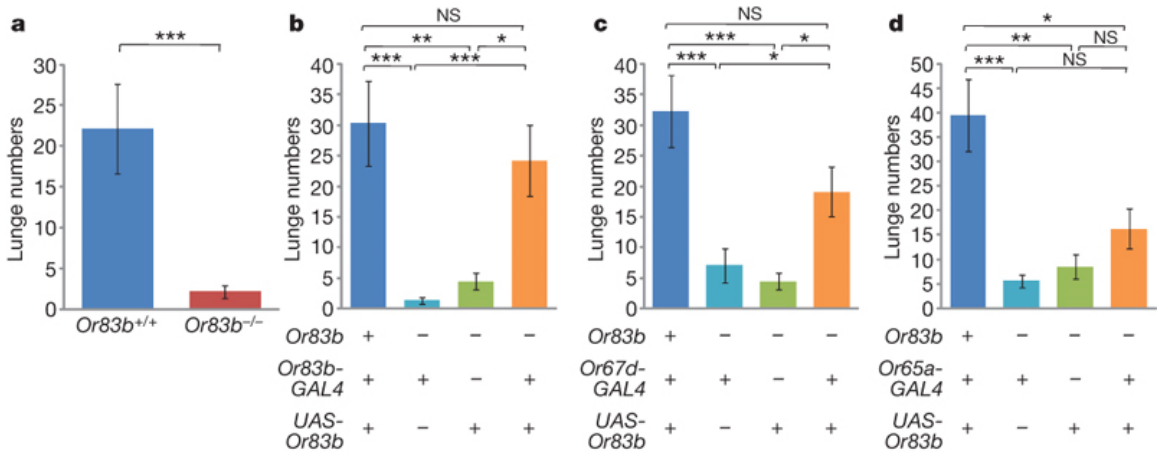
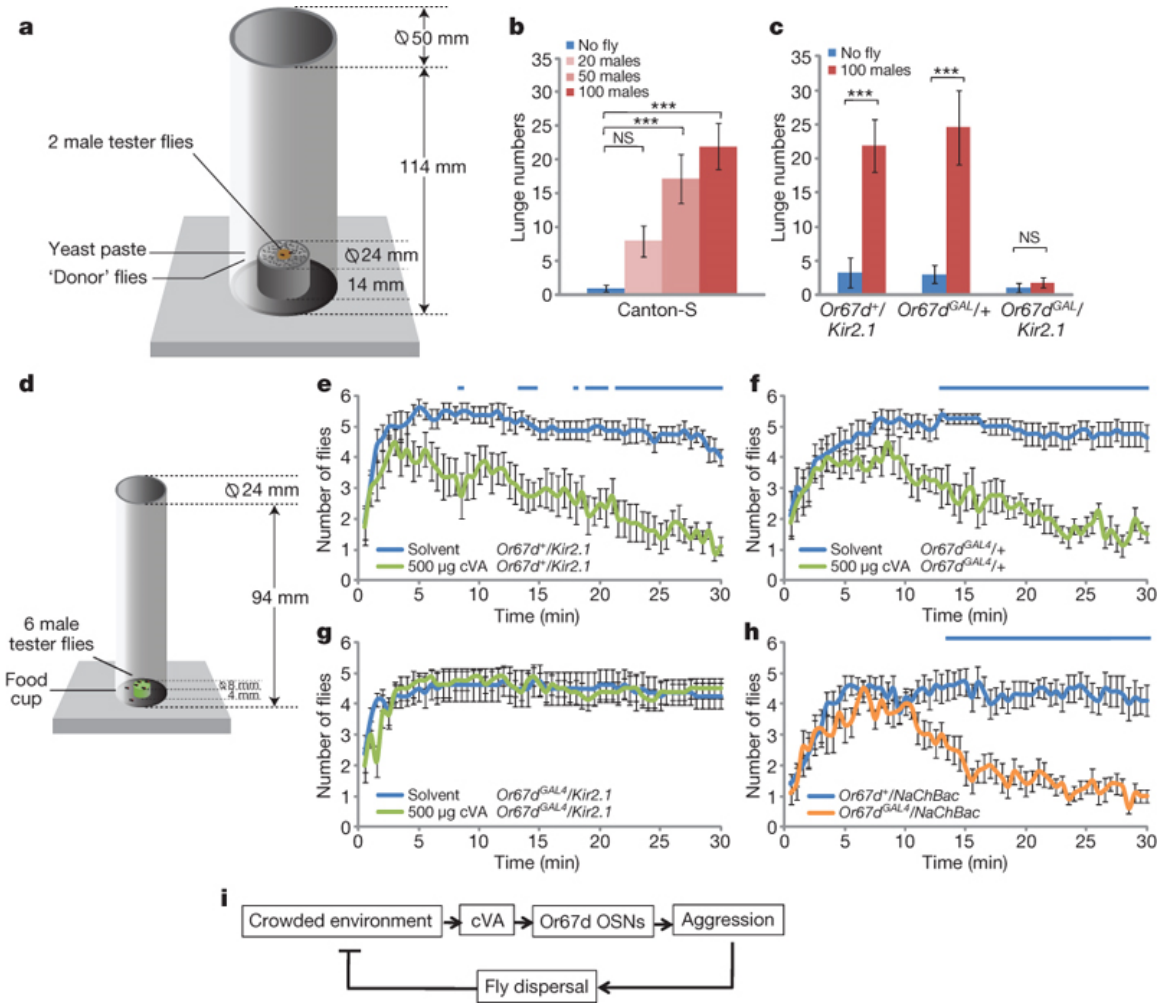

a
b
c
d
Lunge numbers
***
NS
**
*
Or83b+/+
Or83b−/−
Or83b
Or83b-GAL4
UAS-Or83b
Or67d-GAL4
Or65a-GAL4

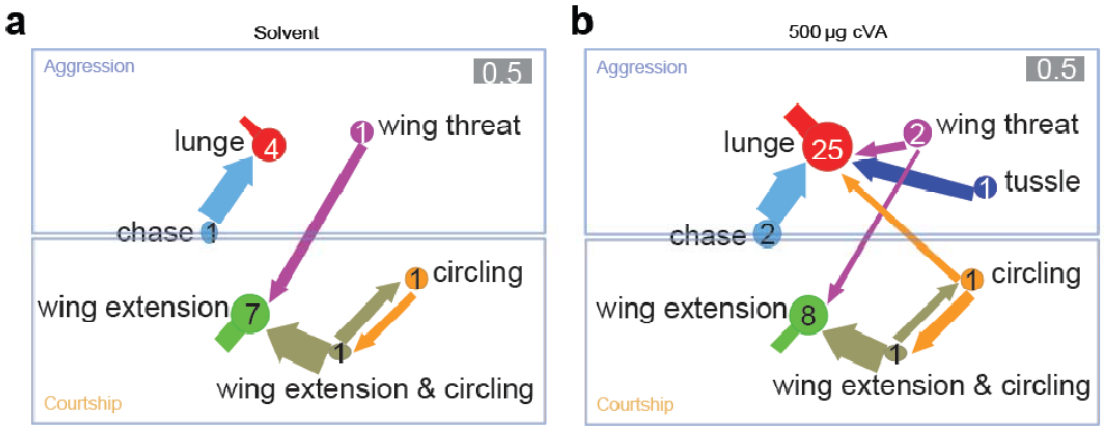
Figure 4. cVA promotes aggression at high fly densities and dispersal of male flies from a food resource

(a) Illustration (to scale) of the behavior chamber used for the experiments shown in (b,c). (b) Number of lunges (per 20 minutes) performed by pairs of "tester" Canton-S males together with the indicated number of caged male "donor" flies (n=15); (c) Number of lunges (per 20 minutes) performed by pairs of "tester" males of the indicated genotype, in the presence or absence of 100 male "donor" flies (n=15). *** $P<0.001$. (d) Illustration (to scale) of the behavior chamber used for the experiments shown in (e-h). (e-h) Number of flies of the indicated genotype on the food cup in the presence of solvent only (blue line) or 500 μg cVA (green line) (n=8). Note gradual dispersal of flies from the food cup in the presence of cVA. Blue bars represent data sets that are significantly different ($P<0.05$). (h) Flies of the indicated genotype tested in the absence of solvent or cVA (n=10). (i) Model illustrating hypothetical negative feedback regulation of fly population density by cVA-promoted aggression.


a
⌀50 mm
2 male tester flies
114 mm
Yeast paste
'Donor' flies
⌀24 mm
14 mm
b
No fly
20 males
50 males
100 males
***
***
NS
Lunge numbers
Canton-S
c
No fly
100 males
***
***
NS
Lunge numbers
Or67d+/ Kir2.1
Or67dGAL/+
Or67dGAL/ Kir2.1
d
⌀24 mm
94 mm
6 male tester flies
Food cup
5 mm
4 mm
e
Number of flies
Solvent Or67d+/Kir2.1
500 µg cVA Or67d+/Kir2.1
Time (min)
f
Number of flies
Solvent Or67dGAL4/+
500 µg cVA Or67dGAL4/+
Time (min)
g
Number of flies
Solvent Or67dGAL4/Kir2.1
500 µg cVA Or67dGAL4/Kir2.1
Time (min)
h
Number of flies
Or67d+/NaChBac
Or67dGAL4/NaChBac
Time (min)
i
Crowded environment
cVA
Or67d OSNs
Aggression
Fly dispersal

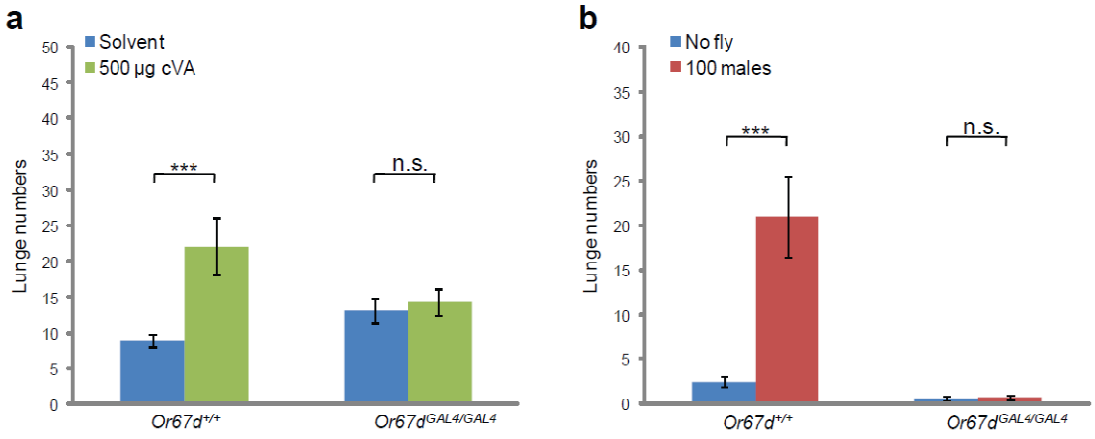
Supplementary Figure 1. Ethograms of fly behaviors upon cVA application.

Ethograms of group-housed Canton-S male flies in the presence of solvent only (a) or 500 µg synthetic cVA (b) showing the transitions between fly behaviors within 10 seconds. The thickness of the arrows represents transition probabilities (grey scale bar: probability=0.5). Arrow stumps represent transitions within the same behavior. Diameters of the circles (log scaled) and the numbers in the circles represent frequencies of behaviors.



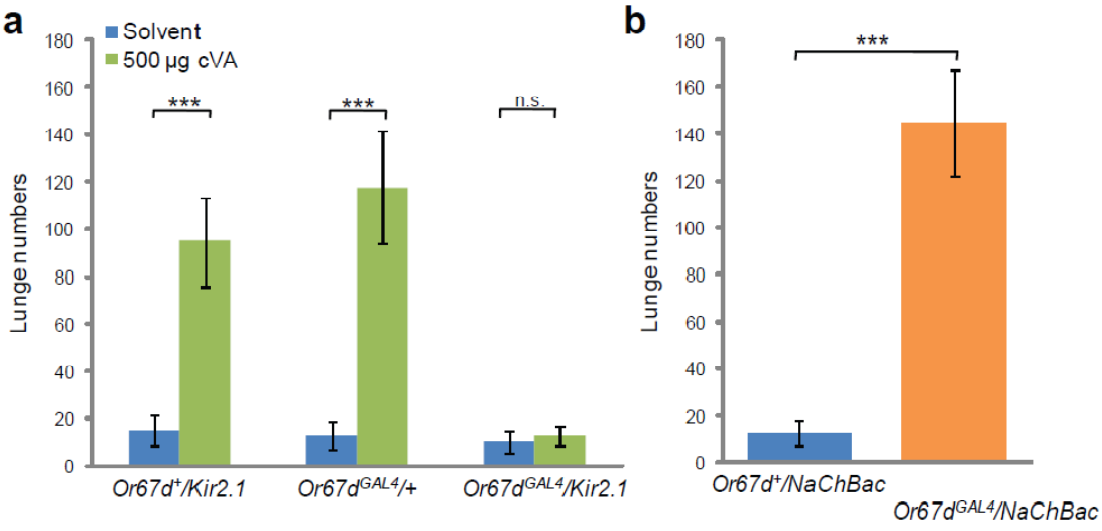
Supplementary Figure 2. The *Or67d* gene is required for the aggression-promoting effect of both synthetic and endogenous cVA.

(a) Number of lunges (per 20 minutes) performed by pairs of mutant $Or67d^{GAL4/GAL4}$ males and $Or67d^{+/+}$ control males, in the presence of solvent only (blue) or 500 µg cVA (green) (n=20-22). The experiments were performed in the setup shown in Fig. 1g. (b) Number of lunges (per 20 minutes) performed by pairs of mutant $Or67d^{GAL4/GAL4}$ males and $Or67d^{+/+}$ control males, in the presence of no donors (blue), or 100 caged male "donor" flies (red) (n=10). The experiments were performed in the setup shown in Fig. 4a. *** P<0.001. Error bars are s.e.m. Note that due to the low aggression level of the genetic background of the mutants, single-housed flies were used for both experiments.



Supplementary Figure 3. Activation of Or67d-expressing OSNs promotes aggression in dispersal assay.

(a) Number of lunges (per 30 minutes) performed by six males of the indicated genotype in the presence of solvent only (blue) or 500 µg cVA (green) (n=8). (b) Number of lunges (per 30 minutes) performed by six males of the indicated genotype (n=10). The experiments were performed in the behavior chamber shown in Fig. 4d. *** P<0.001. Error bars are s.e.m.



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Chapter 4

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

Liming Wang, Xiaoqing Han, Jennifer Mehren, Makoto Hiroi, Jean-Christophe Billeter, Tetsuya Miyamoto, Hubert Amrein, Joel D. Levine, and David J. Anderson

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.

INTRODUCTION

Social interactions between male *Drosophila* involve relatively high levels of aggression, and low levels of courtship (Dankert et al., 2009; Vrontou et al., 2006). 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 (Krstic et al., 2009), specific gustatory receptors (Miyamoto and Amrein, 2008; Moon et al., 2009), or their normal complement of CHs (Billeter et al., 2009; Savarit et al., 1999), non-volatile pheromones that include ligands of gustatory receptors (Ferveur, 2005), 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 (Billeter et al., 2009) has recently been suggested to reduce male-male aggression (Fernández et al., 2010). 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* (Kurtovic et al., 2007), a receptor for cVA (Ha and Smith, 2006; Kurtovic et al., 2007; van der Goes van Naters and Carlson, 2007), 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 (Krstic et al., 2009), and exogenously applied cVA does not reduce male-male courtship (Wang and Anderson, 2010). In contrast, cVA strongly promotes male-male aggression, in an *Or67d*-dependent manner (Wang and Anderson, 2010). 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 (Wang and Anderson, 2010).

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 (Billeter et al., 2009; Krupp et al., 2008). Male oenocytes can be selectively ablated in adults using genetic tools (Billeter et al., 2009) (see Methods). This manipulation eliminated most if not all male CHs (Billeter et al., 2009), including the two most abundant male-enriched CH molecules (Everaerts et al., 2010; Ferveur, 2005; Jallon, 1984) (7-T and (*Z*)-7-pentacosene, 7-P), without affecting the levels of cVA, an olfactory pheromone synthesized in the male ejaculatory bulb (Butterworth, 1969) (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(Billeter et al., 2009) (Supplementary Fig. 3b). However, aggression between such oe^{-} males was not completely eliminated, consistent with a recent report (Fernández et al., 2010).

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 (Chen et al., 2002; Dow and von Schilcher, 1975; Vrontou et al., 2006), 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 (Dankert et al., 2009) (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 (Savarit et al., 1999) (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 (Wang and Anderson, 2010), 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 (Billeter et al., 2009; Ferveur, 2005) (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 (Ferveur, 2005; Jallon, 1984); among these, 7-T has been shown to suppress courtship (Billeter et al., 2009; Lacaille et al., 2007). 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 (Ferveur, 2005), 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 (Lacaille et al., 2007), which express multiple gustatory receptors (Thorne et al., 2004; Wang et al., 2004; Weiss et al., 2011), but no specific receptor for 7-T has yet been identified. The fact that Gr32a is expressed in bitter-sensing GRNs (Lee et al., 2010; Marella et al., 2006; Thorne et al., 2004; Wang et al., 2004; Weiss et al., 2011), and that *Gr32a*$^{-/-}$ mutant males exhibit increased courtship towards decapitated males (Miyamoto and Amrein, 2008), 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 (Miyamoto and Amrein, 2008) 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

(Miyamoto and Amrein, 2008) (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 (Miyamoto and Amrein, 2008), a result that we independently replicated (Supplementary Fig. 9), such decapitated male targets may fail to provide additional signals, such as behavioral feedback (Krstic et al., 2009), 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 (Koganezawa et al., 2010), 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 (Lacaille et al., 2007) is an interesting question that remains to be investigated.

Notably, as fly GRNs often co-express >1 gustatory receptor (Thorne et al., 2004; Wang et al., 2004; Weiss et al., 2011), and as detection of bitter compounds in flies may require multiple receptors (Lee et al., 2010; Lee et al., 2009), 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 (Yew et al., 2009)), and receptor(s) other than Gr32a (Moon et al., 2009), 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 (Caterina et al., 1997) previously used to activate *Drosophila* GRNs (Marella et al., 2006), 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 (Wang et al., 2004) in only a subset of all $Gr32a^{+}$ GRNs(Weiss et al., 2011) (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 (Wang and Anderson, 2010). 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 (Ejima et al., 2007; Kurtovic et al., 2007) 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 (Kurtovic et al., 2007) and are insensitive to its aggression-promoting effect (Wang and Anderson, 2010). 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 (Wang and Anderson, 2010), 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 (Wang and Anderson, 2010). 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 (van der Goes van Naters and Carlson, 2007). Or47b^{+} ORNs express Fru^{M} and project to a sexually dimorphic glomerulus, VA1lm (also known as VA1v) (Couto et al., 2005; Fishilevich and Vosshall, 2005), 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 (Root et al., 2008). 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 (Couto et al., 2005; Fishilevich and Vosshall, 2005) 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 (Billeter et al., 2009; Savarit et al., 1999) (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 (van der Goes van Naters and Carlson, 2007). 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 (Billeter et al., 2009) (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 (Fernández et al., 2010). However, in the case of male-male social interactions, while oenocyte ablation increases male-male courtship (Billeter et al., 2009) it does not fully eliminate male-male aggression (Fig. 1b, c). Since male aggression towards normal females almost never occurs (Fernández et al., 2010), 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 (Issa and Edwards, 2006), as it does in some human populations (Wolff and Jing, 2009).

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 (Dulac and Torello, 2003). A mutation in *TrpC2*, which impairs the detection of pheromones by the vomeronasal organ (VNO (Mombaerts, 2004)), results in both decreased male-male aggression and increased male-male courtship (Leypold et al., 2002; Stowers et al., 2002). By contrast, mutations affecting the main olfactory epithelium (MOE) reduce male-male aggression without increasing inter-male courtship (Mandiyan et al., 2005; Yoon et al., 2005). 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 (Touhara and Vosshall, 2009). Major urinary proteins

(MUPs) have recently been shown to function as murine aggression pheromones, and are detected by a subset of VNO neurons (Chamero et al., 2007). 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.

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FIGURES

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 ***$p<0.001$.

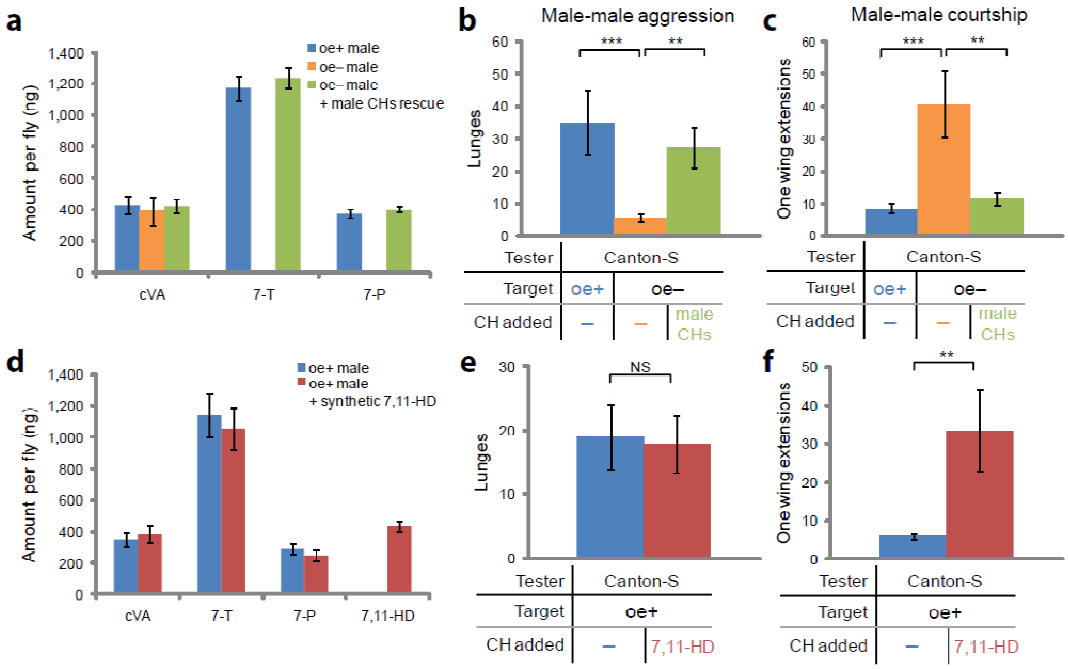
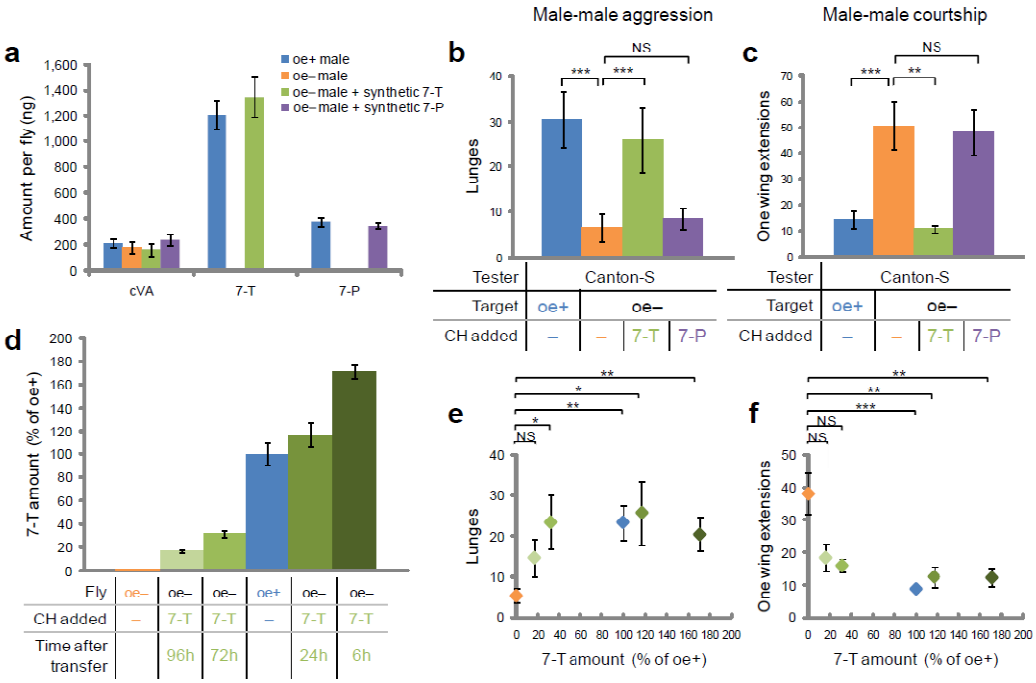

a
Amount per fly (ng)
oe+ male
oe– male
oe– male
+ male CHs rescue
cVA
7-T
7-P
b
Male-male aggression
Lunges
Tester
Canton-S
Target
oe+
oe–
CH added
male CHs
c
Male-male courtship
One wing extensions
d
oe+ male
oe+ male
+ synthetic 7,11-HD
7,11-HD
e
NS
f

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 ***$p<0.001$.


a
Amount per fly (ng)
oe+ male
oe– male
oe– male + synthetic 7-T
oe– male + synthetic 7-P
cVA
7-T
7-P
b
Male-male aggression
Lunges
NS
***
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Tester
Canton-S
Target
oe+
oe–
CH added
–
–
7-T
7-P
c
Male-male courtship
One wing extensions
NS
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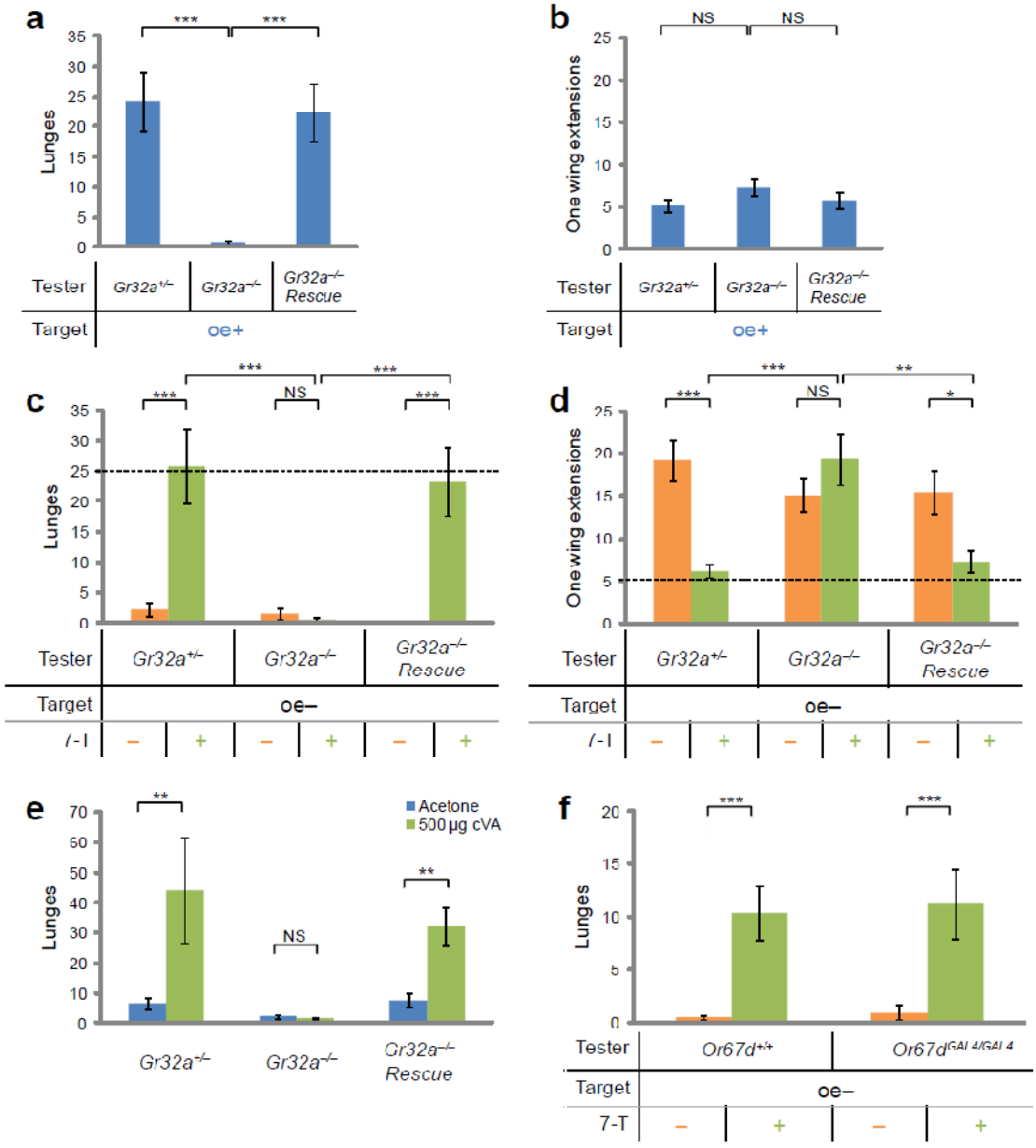
Tester
Canton-S
Target
oe+
oe–
CH added
–
–
7-T
7-P
d
7-T amount (% of oe+)
Fly
oe–
oe–
oe–
oe+
oe–
oe–
CH added
–
7-T
7-T
–
7-T
7-T
Time after transfer
96h
72h
24h
6h
e
Lunges
7-T amount (% of oe+)
f
One wing extensions
7-T amount (% of oe+)

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 ***$p<0.001$.


a
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Lunges
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Tester
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Gr32a-/- Rescue
Target
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One wing extensions
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Gr32a-/-
Gr32a-/- Rescue
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Gr32a-/- Rescue
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+
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+
e
**
Acetone
500 µg cVA
Lunges
70
60
50
40
30
20
10
0
NS
**
Gr32a-/-
Gr32a-/-
Gr32a-/- Rescue
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Lunges
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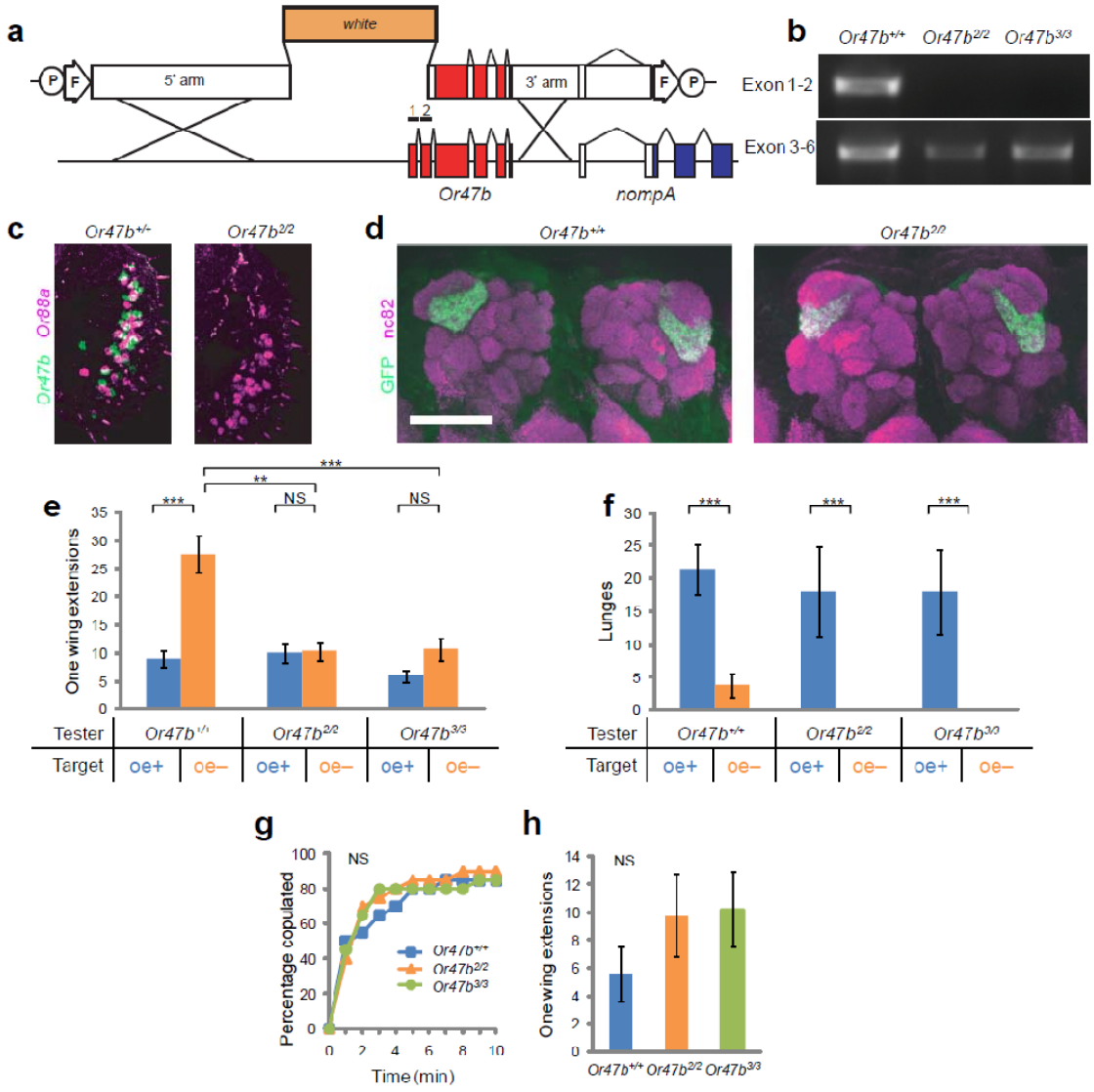
5
0
Tester
Or67d+/+
Or67dGAL4/GAL4
Target
oe–
7-T
–
+
–
+

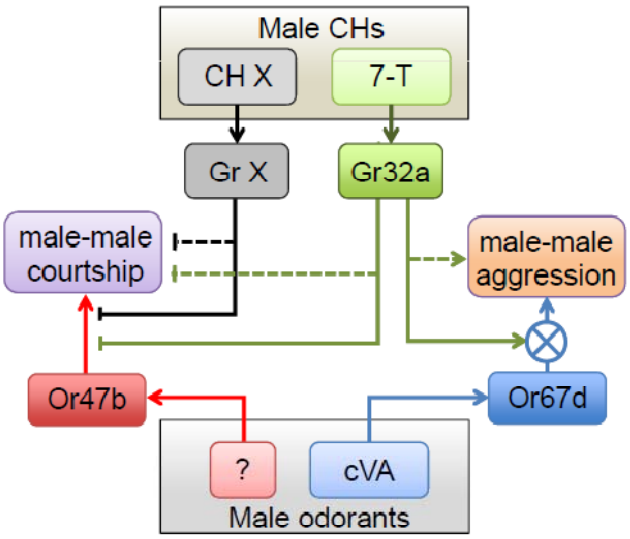
Figure 4. Or47b is required for elevated male-male courtship caused by depletion of male CHs

(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 ***$p<0.001$.


a
white
5' arm
3' arm
P
F
1 2
Or47b
nompA
b
Or47b+/+ Or47b2/2 Or47b3/3
Exon 1-2
Exon 3-6
c
Or47b+/+
Or47b2/2
Or88a
Or47b
d
Or47b+/+
Or47b2/2
nc82
GFP
e
One wing extensions
***
**
NS
NS
Tester
Or47b+/+
Or47b2/2
Or47b3/3
Target
oe+
oe–
f
Lunges
***
***
***
Tester
Or47b+/+
Or47b2/2
Or47b3/3
Target
oe+
oe–
g
Percentage copulated
NS
Or47b+/+
Or47b2/2
Or47b3/3
Time (min)
h
Onewing extensions
NS
Or47b+/+ Or47b2/2 Or47b3/3

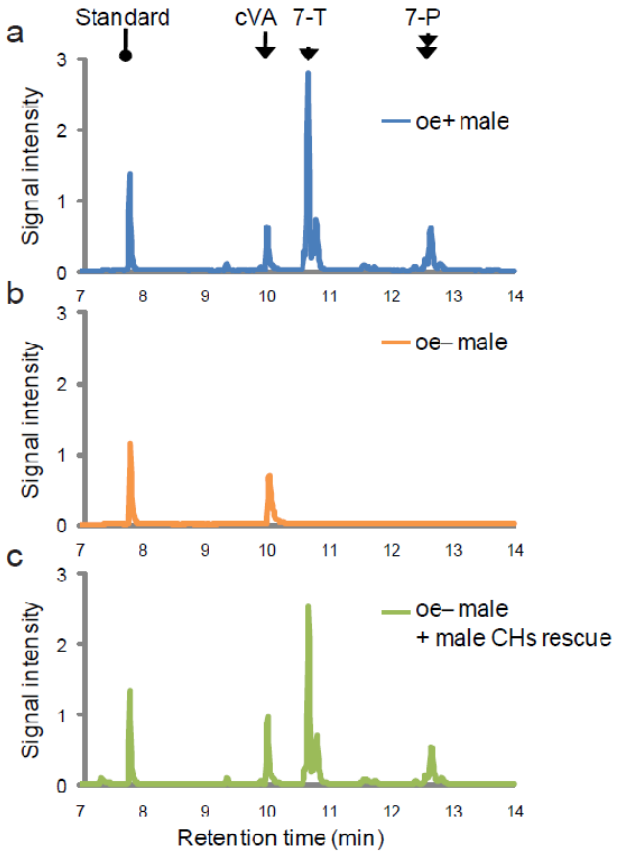
Supplementary Fig. 1. Diagram illustrating the interactions between gustatory and olfactory pheromones that control male social behaviors.

The requirement of 7-T/Gr32a for the aggression-promoting influence of cVA may represent an indirect, permissive effect (solid arrow), or may involve more direct sensory gating (dashed arrow). Similar alternative mechanisms could explain the effect of male CHs to suppress the courtship-promoting effect of an unknown pheromone(s) ("?") detected by Or47b. "CH X/Gr X" indicates additional gustatory pheromones/receptors that redundantly suppress male-male courtship.



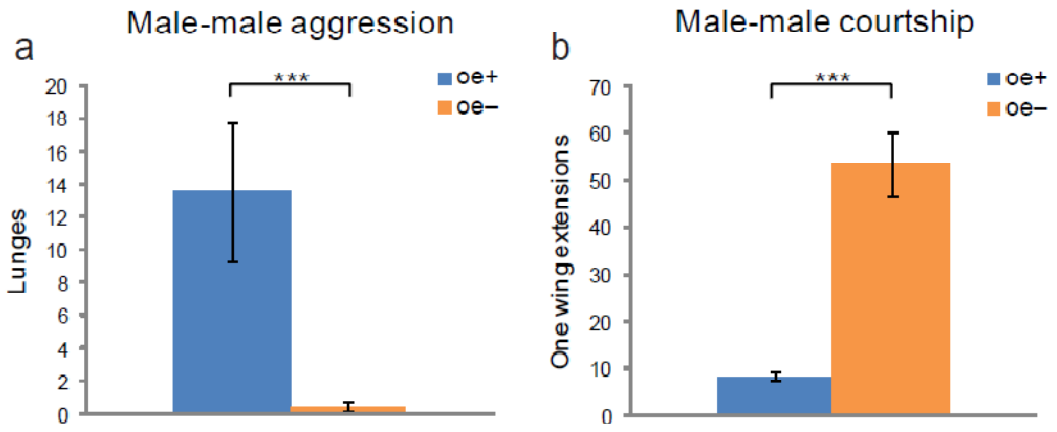
Supplementary Fig. 2. Ablation of male oenocytes eliminates male CHs.

Representative gas chromatography traces of CHs from individual oe^{+} (a, blue), oe^{-} (b, orange) or oe^{-} males perfumed with male CHs (c, green). Arrows in (a) indicate the internal standard and representative pheromone peaks. Quantification of major pheromones peaks is shown in Fig. 1a. In this figure or following figures, the oe^{+} and oe^{-} males were generated by crossing male *+; PromE(800)-GAL4, tub-GAL80^{TS}; +* with female *+; UAS-StingerII; +* or *+; UAS-StingerII, UAS-hid/CyO; +*, respectively (see the Methods section for details).



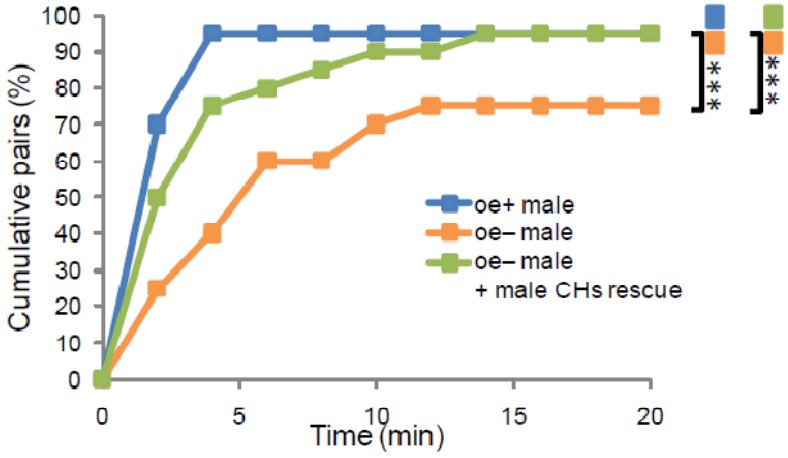
Supplementary Fig. 3. Male CHs are important for the normal balance of male-male social behaviors.

Quantification of aggression (a) and courtship (b) performed by pairs of oe^{+} (blue) or oe^{-} (orange) tester males (n=20). Tester males were single-housed 5–7 days before behavioral assays. Error bars are s.e.m. in this and all following supplementary figures. ***p<0.001.



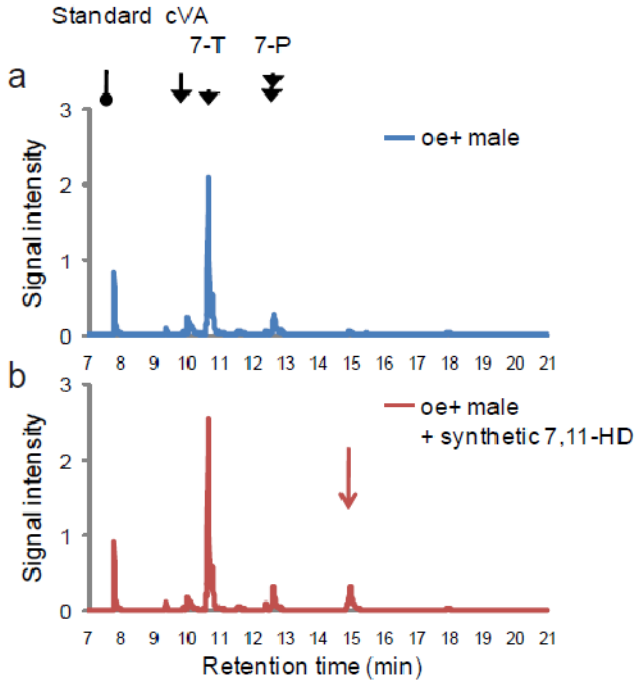
Supplementary Fig. 4. Elimination of male CHs delays the onset of male-male aggression.

Cumulative percentage of pairs that exhibit any lunge by the Canton-S tester flies at a given time point, towards oe^{+} (blue), oe^{-} (orange), or oe^{-} target males perfumed with male CHs (green) (n=20). The down-shift of the orange curve suggests the increase of latency to the first lunge. ***p<0.001.



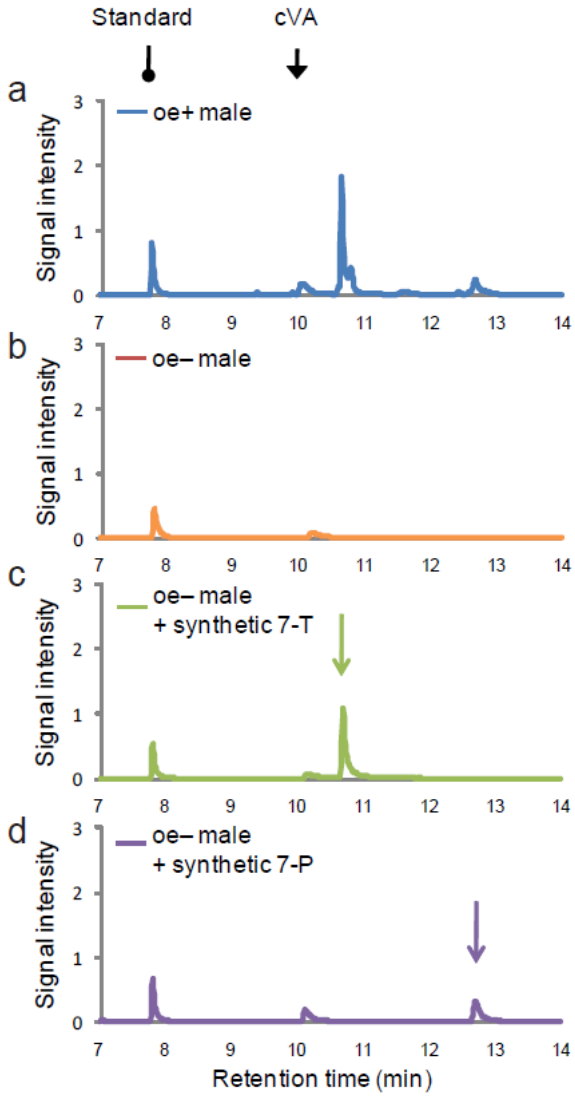
Supplementary Fig. 5. Perfuming oe^{+} males with synthetic 7,11-HD.

Representative gas chromatography traces of CHs from individual oe^{+} males (a, blue) or oe^{+} males perfumed with synthetic 7,11-HD (b, red). Arrow in (b) indicates the 7,11-HD peak. Quantification of major pheromone peaks is shown in Fig. 1d.

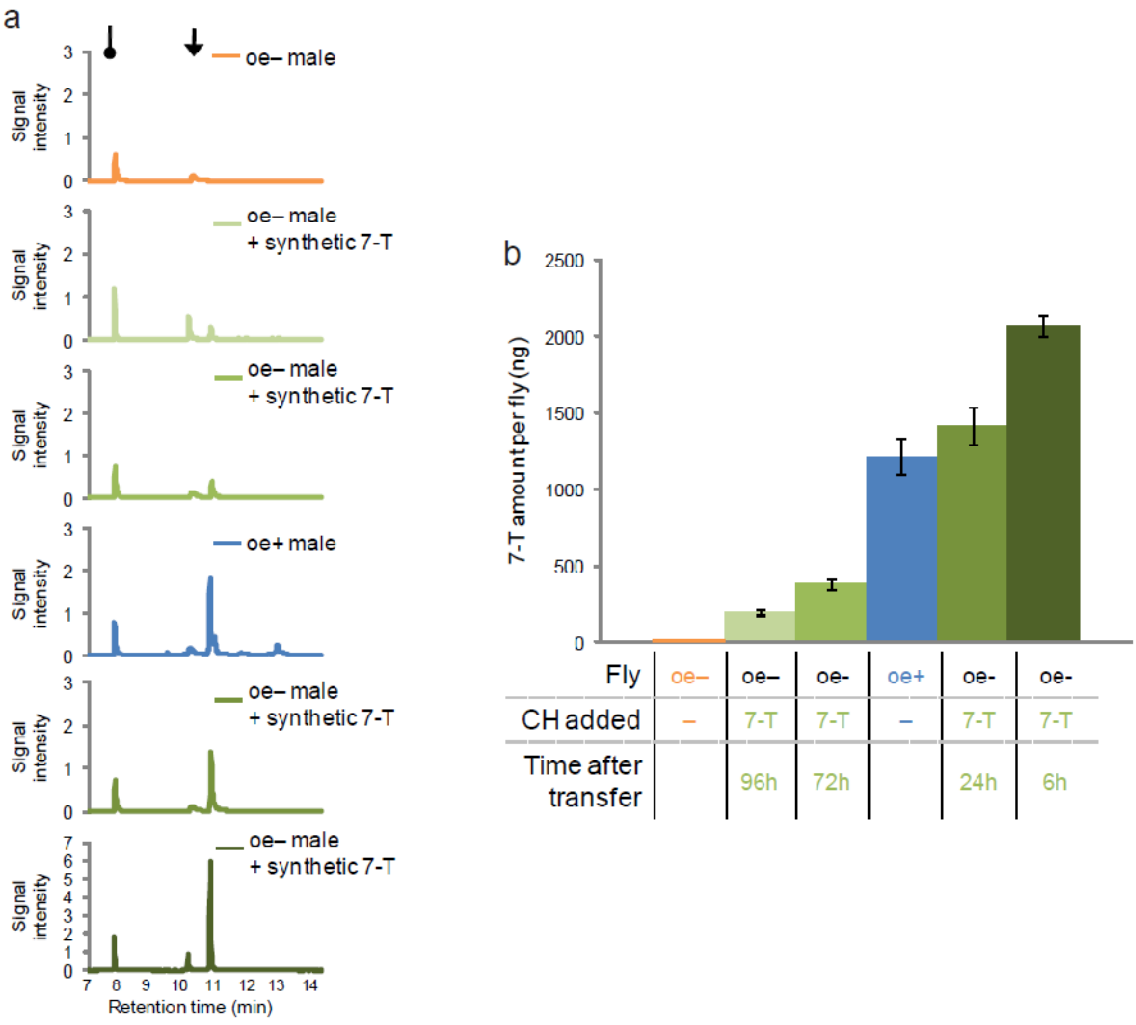


Supplementary Fig. 6. Perfuming oe$^-$ males with synthetic 7-T and 7-P.

Representative gas chromatography traces of CHs from individual oe$^+$ (a, blue), oe$^-$ (b, orange), or oe$^-$ males perfumed with synthetic 7-T (c, green) or 7-P (d, purple). Arrows indicate 7-T (c) and 7-P (d) peaks. Quantification of major pheromone peaks is shown in Fig. 2a.

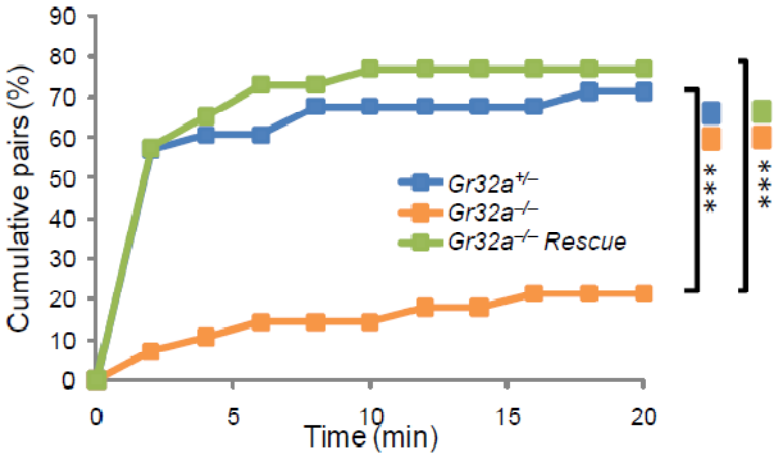


Supplementary Fig. 7. Perfuming oe$^-$ males with different amounts of synthetic 7-T. (a) Representative gas chromatography traces of CHs from individual oe$^-$ (orange), oe$^+$ (blue) or oe$^-$ males perfumed with various amounts of synthetic 7-T (green). Male oe$^-$ flies were incubated after 7-T transfer for various periods of time. Green shading represents relative amount of 7-T carried by oe$^-$ males (darker=higher). (b) Quantification of 7-T from male flies (n=9–11). Fly conditions as in (a). Note the oe$^+$ data were the same as in Fig. 2a. The relative quantification of this data set was shown in Fig. 2d.



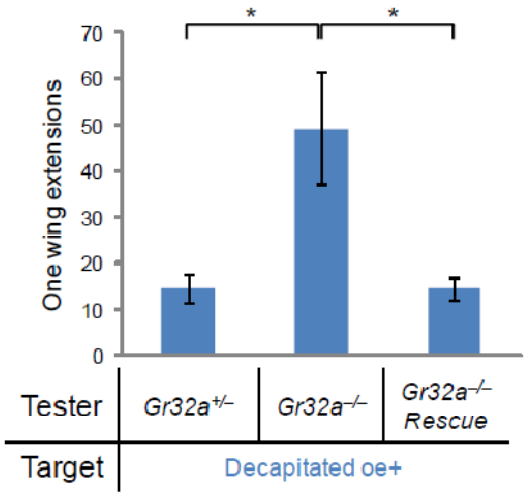
Supplementary Fig. 8. Elimination of *Gr32a* delays the onset of male aggression.

Cumulative percentage of pairs that exhibit any lunge by tester flies of the indicated genotypes at a given time point, towards oe^{+} target males (n=26–28). The down-shift of the orange curve suggests the increase of latency to the first lunge. ***p<0.001.



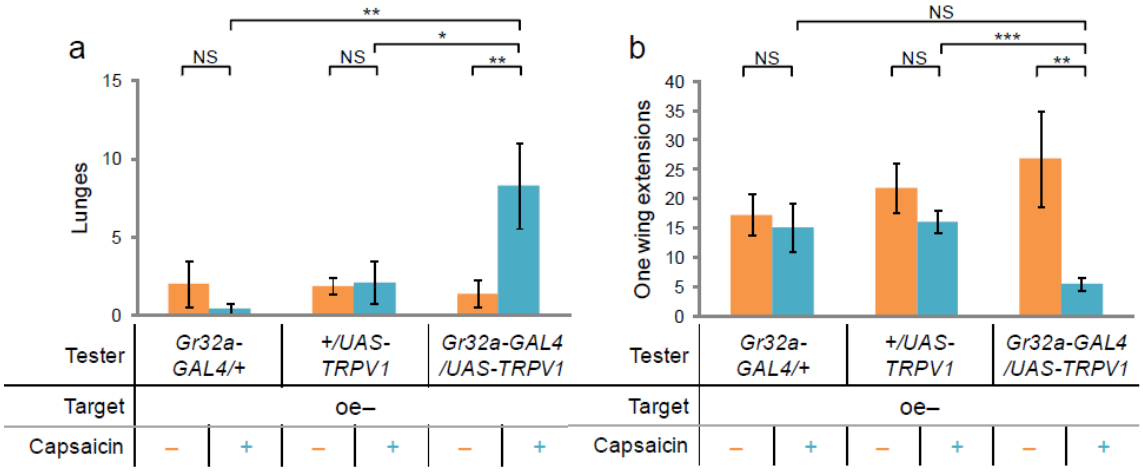
Supplementary Fig. 9. *Gr32a* is required for the suppression of courtship towards decapitated males.

Quantification of courtship performed by male flies of the indicated genotypes towards decapitated oe^{+} target males (n=18–20). The experimental setup was identical as Fig. 3a, b, except that the target males were decapitated and placed in the center of the behavior arena before assays. *p<0.05.



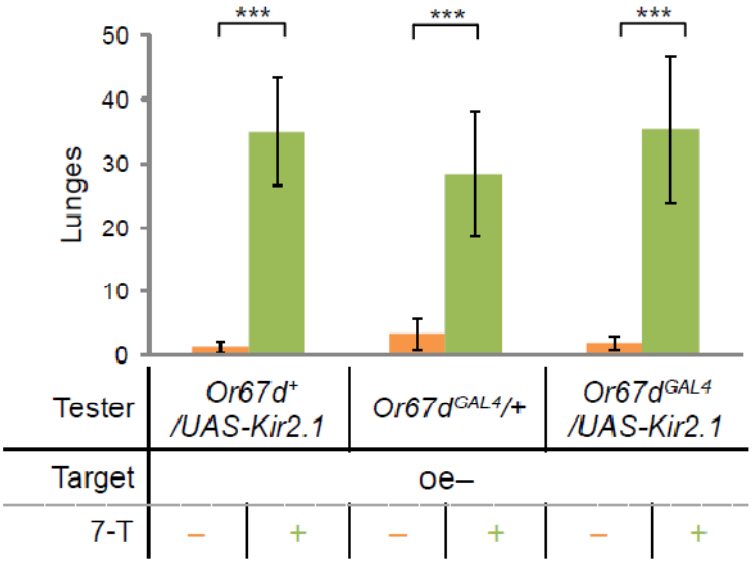
Supplementary Fig. 10. Activating Gr32a^{+} GRNs partially mimics the behavioral effects of 7-T.

Quantification of aggression (a) and courtship (b) performed by males of the indicated genotypes towards oe^{-} tester males (orange) or oe^{-} males carrying capsaicin (aqua) (n=20). NS, p>0.05, *p<0.05, **p<0,01, ***p<0.001. Note in this experiment, flies were aged for ~12–14 d prior to behavioral assays, to ensure adequate expression of GAL4 protein.



Supplementary Fig. 11. Or67d^{+} ORNs are not required for the aggression promoting effect of 7-T.

Quantification of aggression performed by male flies of the indicated genotypes towards oe^{-} target males (orange) or oe^{-} males perfumed with 7-T (green) (n=18). ***p<0.001.



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 (Miyamoto and Amrein, 2008) 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 (Billeter et al., 2009) 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* (Zhou et al., 1997) in oenocytes specifically in the adult stage (under the control of *tub-GAL80*^{TS}) (McGuire et al., 2003) 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 (Dankert et al., 2009; Wang et al., 2008). 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 (Dankert et al., 2009). 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 (Dankert et al., 2009; Hoyer et al., 2008). Briefly, a rectangular chamber (4 cm X 5 cm X 12 cm) was placed on top of an acrylic base. In the center of the base, a 1 cm X 1 cm X 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 (Wang and Anderson, 2010). 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 (Davis and Carlson, 1989; Wenkert et al., 1985). 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 (Billeter et al., 2009; Krupp et al., 2008). 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 x 0.25 mm ID x 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 (Savarit et al., 1999). 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 (Billeter et al., 2009). 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. (Marella et al., 2006)). 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 (Gong and Golic, 2003), 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.

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Chapter 5

Conclusions and future directions

Social experience and fly behaviors

In Chapter 2, I showed that long-term (i.e., in the period of days) social experience with conspecific males suppressed male aggression in *Drosophila* in a reversible manner. Furthermore, this robust and reversible behavioral effect allowed me to identify genetic components underlying the social regulation of male-male aggression (Wang et al., 2008).

I would like to point out here that social experience has been shown to influence multiple animal behaviors besides aggression. And there is unlikely to be a generic mechanism that underlies the social regulation of all animal behaviors. How social experience regulats behaviors is an interesting direction for future studies.

In the fruit fly, social experience also influences courtship behavior of male flies (Dankert et al., 2009). Group-housed male flies show reduced courtship towards decapitated virgin females, measured by the occurrence of one wing extension, a typical courtship behavior. The mechanism of the social suppression of courtship is not clear. Besides social behaviors, social experience also influences multiple non-interactive behaviors. Group-housed flies synchronize their circadian rhythm (measured by locomotor activity) (Levine et al., 2002), and increase daytime sleep (Ganguly-Fitzgerald et al., 2006). Both effects seem to be chemosensory system–dependent. It is therefore an intriguing question how social experience modulates fly behaviors: it may down-regulate the arousal state of fruit flies, which in turn suppresses multiple behaviors that rely on arousal state, including aggression, courtship, and sleep; or it may suppress multiple behaviors directly through different mechanisms.

One way to approach this question is to ask whether there is a condition or manipulation that disassociates the effect of social experience on different behaviors. It may be an interesting idea to perform unbiased genetic or neural circuitry screens (GAL4 based) to identify genes and neuronal populations that underlie the social regulation of different behaviors. It will provide a comprehensive understanding of how social experience regulates different behaviors, and whether there is any general "principle" (e.g., genetic network, neural circuitry, etc.) that mediates the regulation of various animal behaviors.

Another possible approach is to examine the functions of neurotransmitters, especially biogenic amines. Biogenic amines, such as dopamine (DA), serotonin (5-HT), octopamine (OA), tyramine (TA), and histamine (HA), have been shown to modulate various developmental and physiological processes in the fruit fly, including behaviors. For example, OA system has been shown to be necessary for male aggression and female ovulation (Certel et al., 2007; Hoyer et al., 2008; Monastirioti et al., 1996; Zhou et al., 2008), as well as appetitive learning (Schwaerzel et al., 2003) and sleep (Crocker et al., 2010). And DA modulates a variety of fly behaviors, including learning and memory (Claridge-Chang et al., 2009; Krashes et al., 2009; Schwaerzel et al., 2003; Zhang et al., 2007), arousal state (Andretic et al., 2005; Kume et al., 2005; Lebestky et al., 2009), locomotion (Kong et al., 2010), and social behaviors (Liu et al., 2008, 2009). Notably, activating the OA system reverses the social suppression of aggression (Zhou et al., 2008) (however, it is not clear whether such effect is only seen in group-housed flies or not); and DA is involved in the social promotion of daytime sleep (Ganguly-Fitzgerald et al., 2006). Therefore, it is a reasonable assumption that biogenic amines play important roles

in mediating the social regulation of fly behaviors. I propose that by studying which and how biogenic amines mediate the effect of social experience on different behaviors, we will gain a better understanding about how social experience influences multiple aspects of animal lives.

Cyp6a20 underlying the social regulation of fly aggression

In Chapter 2, I showed that *Cyp6a20*, a previously identified aggression suppressing gene (Dierick and Greenspan, 2006), mediates the suppression of aggression by social experience in the fruit fly (Wang et al., 2008). The discovery of *Cyp6a20* as an aggression-suppressing gene and its enriched expression in pheromone sensing olfactory organs led to the discovery of *cis*-11-vaccenyl acetate (cVA), the first identified aggression-promoting pheromone in the fruit fly (Chapter 3) (Wang and Anderson, 2010). However, it is not clear how this gene mediates the social suppression of fly aggression.

One attractive hypothesis, as I discussed in Chapter 2, is that *Cyp6a20* may regulate the sensitivity of aggression-regulating pheromones (e.g., cVA) upon social experience. *Cyp6a20* encodes a cytochrome P450 enzyme, a member of a large and diverse group of enzymes that are present in all domains of life (Danielson, 2002; Schuler, 2011). Cytochrome P450s metabolize a broad variety of substrates, endogenous (e.g., lipids and steroid hormones) or exogenous (e.g., drugs and pesticides). Notably, cytochrome P450s have been implicated in the normal function of chemosensory systems in insects. Multiple cytochrome P450s have been shown to be expressed in insect

antennae, where the olfactory receptor neurons (ORNs) are located (Hovemann et al., 1997; Maïbèche-Coisne et al., 2005; Maïbèche-Coisne et al., 2004; Wang et al., 1999; Wojtasek and Leal, 1999). In particular, antennal cytochrome P450s of the pale-brown chafer P. diversa metabolize and degrade species-specific pheromones *in vitro* (Wojtasek and Leal, 1999), and inhibiting cytochrome P450s in the antennae of *P. diversa* desensitizes pheromonal responses in ORNs (Maïbèche-Coisne et al., 2004). Taken together, these data suggest that cytochrome P450s may regulate sensitivity of insect chemosensory systems by degrading pheromones: they may clear up excess amount of pheromones in the extracellular lymph of ORNs to permit the acute detection of varying pheromone concentration (Maïbèche-Coisne et al., 2004); alternatively they may degrade pheromones to decrease their sensitivity (Dierick and Greenspan, 2006).

Therefore I hypothesize that *Cyp6a20* may mediate the aggression-suppressing effect of social experience in male flies by degrading cVA (and/or other aggression-promoting pheromones) and suppressing their sensitivity in group-housed males. There are three sets of experiments we can do to test this hypothesis: A) Does social experience regulate olfactory sensitivity? The most straightforward experiment will be to record from the ORNs of single- vs. group-housed male flies, and ask whether cVA (or the crude mixture of male fly odors) elicits differential responses. We can also use behavioral responses to probe pheromonal sensitivity; e.g., whether single-housed flies show higher sensitivity to the aggression-promoting and/or courtship-suppressing effects of cVA, etc. If A) is true, then B) becomes is *Cyp6a20* involved in the social regulation of pheromonal sensitivity? This question can also be asked in an electrophysiological or behavioral setting, or both. Alternatively, we can test C) Can *Cyp6a20* degrade cVA or other

pheromones under *in vitro* or *ex vivo* conditions? And if so, does suppressing such enzymatic activity (pharmacologically or genetically) *in vivo* phenocopy *Cyp6a20*$^{-/-}$ mutants in aggression assays?

Notably, although the possible link of social experience, *Cyp6a20* and cVA (and/or other pheromones) sensitivity is a very attractive one, we have no data supporting this hypothesis at all at this moment. Therefore it is important to bear in mind the presence of other plausible mechanisms. It is still possible that the non-antennal expression of *Cyp6a20* (in the abdomen, in the CNS, etc) plays a role in regulating male aggression by a completely different mechanism. Also, cytochrome P450s may be involved in the survival and normal functioning of antennal cells, rather than directly involved in the clearance/degradation of pheromones.

Olfactory and gustatory pheromones in fly social behaviors

In Chapters 3 and 4, we identified at least three classes of pheromones that worked in a hierarchical manner to define appropriate male social behaviors: olfactory pheromones (e.g., cVA via Or67d) that regulated the intensity of male-male aggression; gustatory pheromones (e.g., 7-T via Gr32a) that regulated the gender-specificity of male social behaviors; and some unknown pheromone(s) that promoted male courtship behavior via Or47b. However, it is important to mention here that cVA/7-T/Or47b-ligand probably only represents a small (yet critical) portion of the pheromones that are involved in the regulation of fly social behaviors. Also, the neural basis of phermonal regulation of

fly behaviors is only starting to be uncovered. The identification and characterization of additional pheromones and the underlying neural circuitry may shed important light on the understanding of pheromonal regulation of fly behaviors.

First of all, although cVA is sufficient to promote male-male aggression, lack of cVA sensitivity (either by eliminating its receptor Or67d or by silencing the ORNs expressing Or67d) does not affect baseline aggression between pairs of male flies (Wang and Anderson, 2010). This seeming discrepancy suggests that additional olfactory pheromone(s) may be able to permit male-male aggression even in the absence of cVA detection (Chapter 3). What are these pheromones? And how are they detected? In Chapter 3, I showed that *Or83b*$^{-/-}$ mutant flies, in which ~ 70% of ORNs lose sensitivity (Larsson et al., 2004), had aggression deficit. And rescuing *Or83b* specifically in Or67d^{+} ORNs in *Or83b*$^{-/-}$ background is sufficient (at least partially) to restore aggression. Similarly, we can rescue *Or83b* in other groups of ORNs in *Or83b*$^{-/-}$ background and ask whether and which group of ORNs may also be sufficient to restore aggression. By doing so, we expect to identify additional olfactory receptor(s) and ORNs that are involved in the regulation of male-male aggression, which will also help the identification of any additional olfactory pheromones that regulate aggression. It will be interesting then to examine the interactions among multiple aggression-regulating olfactory pheromones, at both behavioral and circuitry levels.

Similarly, 7-T is only one of the male cuticular hydrocarbons (CHs) identified from male flies. To make the story more complicated, 7-T detection seems to be necessary for the induction of male-male aggression, but not for the suppression of male-

male courtship, suggesting the presence of an additional gustatory pheromone that suppresses male-male courtship (such as CH503 (Yew et al., 2009)) (Chapter 4). It is also puzzling why 7-pentacosene (7-P), the second most abundant male CHs (Everaerts et al., 2010), does not exert any behavioral effect. There is some indirect evidence suggesting 7-P may promote courtship (Savarit and Ferveur, 2002; Sureau and Fervuer, 1999). However direct examination of the behavioral effect of 7-P under different circumstances is needed. In addition, the functions of female-specific CHs such as (*Z,Z*)-7,11-heptacosadiene (7,11-HD) and (*Z,Z*)-7,11-nonacosadiene (7,11-ND) (Ferveur, 2005; Marcillac et al., 2005) are largely unknown. Female CHs seem not to be required for normal male-female courtship (Billeter et al., 2009). Consistent with this idea, male flies lacking gustatory sensilla court normally towards females (Boll and Noll, 2002; Krstic et al., 2009). However, gustatory input like 7,11-HD and 7,11-ND may still play a role in male-female courtship in the absence of other sensory input. For example, in Chapter 4 I showed that 7,11-HD was sufficient to promote courtship towards males; and male flies lacking both olfactory and gustatory inputs show deficit in male-female courtship (Krstic et al., 2009). Therefore, it is of interest to study how female CHs are sensed by males and modulate male behaviors. Furthermore, it is not clear whether female CHs play a role in the regulation of female-female aggression.

One unexpected discovery I showed in Chapter 4 is that male-male courtship is not a default behavioral state, but is rather triggered by pheromone(s) detected by Or47b. Although we haven't identified the ligand(s) of Or47b receptor, the idea that male-male courtship is an induced rather than default behavior itself is very important, given that it argues against the prevailing view in the field. One immediate question is why the male

flies carry a courtship-promoting pheromone. One possibility is that the primary function of Or47b is to recognize conspecifics (*Drosophila melanogater*); and the "default" behavior towards conspecific animals (regardless the sex) is courtship (note: it is not a "default" behavior towards any animals *per se*, but only towards conspecifics). In other words, Or47b ligand(s) is not a courtship-promoting pheromone per se, but a species "tag". And on top of it, male CHs define appropriate male social behaviors towards different sexes of *Drosophila melanogaster* individuals. If this hypothesis is correct, then we would assume that males of other *Drosophila* species will not court *D. melanogaster*, and such courtship suppression will diminish if Or47b signaling is eliminated in these males. This is a testable hypothesis and may lead to a better understanding of the contribution of behaviors in speciation. Alternatively, Or47b signaling may play an active role in defining male-male social interactions. For example, it is possible that male-male courtship (induced by Or47b) may be necessary to bring two male flies in close proximity, which facilitates the detection of short-range, sex-specific pheromones such as 7-T. In this regard, male-male courtship may be the prerequisite of male-male aggression. This is also a plausible hypothesis which can be directly tested by asking whether male-male courtship always precedes male-male aggression and if so whether eliminating Or47b signaling has any effect on male-male aggression under different circumstances.

The interplay between olfactory and gustatory systems

One important implication from Chapter 4 is the hierarchical interactions among three classes of pheromones in the regulation of male social behaviors: male CHs (7-

T/Gr32a) behaviorally gated the aggression-promoting effect of olfactory pheromones (cVA); and male CHs were also epistatic to the courtship-promoting effect of Or47b. However, the circuitry basis of such hierarchical interactions remains unknown.

The fact that Gr32a is required for the aggression-promoting effect of cVA suggests that 7-T circuitry may feed onto the cVA circuitry and regulates its activity. Recent progress in the characterization of cVA circuitry has made it possible to test this hypothesis directly. By doing photoactivatable-GFP (PA-GFP) based tracing, the second- and third-order neurons in cVA circuitry have been identified, with their responses to cVA recorded by both electrophysiology and calcium recording (Datta et al., 2008; Ruta et al., 2010). Strikingly, one male-specific cluster of the third-order neurons located in the dorsal anterior protocerebrum (DC1) may receive input from gustatory system: DC1 dendrites overlap and interdigitate with axonal projections of neurons located in the subesophageal ganglion (SOG), presumptively the gustatory interneurons (Ruta et al., 2010). Therefore, it is possible that the third order cVA-responsive neurons or their downstream targets are modulated by gustatory input such as 7-T. Calcium imaging of cVA responses in cVA circuitry (e.g., DC1 neurons) +/- 7-T stimulation (direct stimulation or neuronal activation by channelrhodopsin) may be the best way to test this hypothesis.

Interestingly, the two male-specific clusters of third-order cVA neurons, DC1 and LC1, have distinct properties. Although both clusters are cVA-responsive, DC1 neurons are excitatory while LC1 are inhibitory ($GABA^{+}$) (Ruta et al., 2010). Coincidently, the opposite effects of cVA on male social behaviors have been reported: an inhibitory effect

on courtship (Kurtovic et al., 2007), and stimulative effect on aggression (Wang and Anderson, 2010). Therefore an attractive hypothesis is that DC1 and LC1 neurons mediate the aggression-promoting and courtship-suppressing effects, respectively. In addition, cVA's aggression-promoting and courtship-suppressing effects are only salient in male-male and male-female interactions, respectively (Wang and Anderson, 2010). Therefore following the same logic, DC1 neurons may dominate the cVA response (aggression-promoting) in the presence of 7-T stimulation, while LC1 may dominate the courtship-suppressing response in the absence of 7-T. Whether this hypothesis is true, and if so how DC1 and LC1 neurons interact in the presence or absence of gustatory input, are both interesting questions remained for future studies.

It is also worth noting that male CHs suppress the courtship-promoting effect of Or47b. The circuitry basis for the interaction between male CHs and Or47b is another interesting question. Like Or67d, Or47b is also expressed in Fru^+ ORNs (Couto et al., 2005; Fishilevich and Vosshall, 2005). It is therefore possible that some Fru^+ interneurons (like DC1 and LC1 neurons) mediate the suppressive effect of male CHs on the Or47b pathway. It may be interesting to dissect Or47b circuitry (using PA-GFP tracing and calcium imaging or electrophysiology) and examine its interaction with gustatory system.

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Appendix

Automated monitoring and analysis of social behavior in *Drosophila*

Heiko Dankert, Liming Wang, Eric D. Hoopfer, David J. Anderson, and Pietro Perona

Automated monitoring and analysis of social behavior in *Drosophila*

Heiko Dankert[1,2], Liming Wang[2], Eric D Hoopfer[2], David J Anderson[2] & Pietro Perona[1]

We introduce a method based on machine vision for automatically measuring aggression and courtship in *Drosophila melanogaster*. The genetic and neural circuit bases of these innate social behaviors are poorly understood. High-throughput behavioral screening in this genetically tractable model organism is a potentially powerful approach, but it is currently very laborious. Our system monitors interacting pairs of flies and computes their location, orientation and wing posture. These features are used for detecting behaviors exhibited during aggression and courtship. Among these, wing threat, lunging and tussling are specific to aggression; circling, wing extension (courtship 'song') and copulation are specific to courtship; locomotion and chasing are common to both. Ethograms may be constructed automatically from these measurements, saving considerable time and effort. This technology should enable large-scale screens for genes and neural circuits controlling courtship and aggression.

How are innate behaviors programmed into the genome? Answering this question requires identifying the genes that control specific behaviors, the neural circuitry on which they act, and how this circuitry controls behavior[1–5]. This may be attempted in model organisms such as the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*, thanks to the abundant genetic tools for marking, mapping and manipulating specific populations of neurons[6,7], thereby enabling large-scale genetic and functional screens[8,9].

Social behaviors, such as courtship and aggression, are of particular interest because they have strong innate components. Mating has been studied in both *D. melanogaster* and *C. elegans* using a combination of molecular genetic and cellular approaches[5,10]. *Drosophila* is unique among invertebrate genetic model organisms in that it exhibits both courtship and aggression[11–14].

Measuring animal behavior is difficult. Both aggression and courtship consist of rich ensembles of stereotyped behaviors, which often unfold in a characteristic sequence[13,15]. Currently these behaviors are measured manually, which is slow and laborious. Subjective decisions by the observer may lead to difficulty in reproducing experiments. Furthermore, human observers may fail to detect behavioral events that are too quick or too slow and may miss events owing to flagging attention. These constitute substantial obstacles to conducting large-scale behavioral screens.

These limitations could be overcome through automation. The first step toward measuring behavior is tracking (that is, measuring the position and orientation of animals over time). Machine vision systems have been designed for tracking houseflies[16], mice[17], ants[18], bees[19], single *Drosophila* in three dimensions[20] and for measuring *Drosophila* locomotion[9,21,22], showing promising accuracy and flexibility. However, we do not yet have systems for measuring complex behaviors automatically. A machine vision apparatus that detects lunging, an aggressive behavior in *Drosophila*, has been developed recently[23]. In addition to lunging, it would be desirable to measure other aggressive behaviors, such as chasing, tussling, boxing (fencing) and wing threat[13], as well as courtship behaviors. This would allow the study of whether a given mutation or environmental influence exerts a selective effect on aggression or on social interactions in general[24].

Here we describe a machine vision system designed to quantify and analyze various social behaviors in *Drosophila*. Actions associated with courtship, aggression and locomotion are detected from overhead videos of fly pairs. We designed our system, first, to allow detailed, accurate and reproducible quantitative measurements of individual component behaviors ('actions') that are expressed in courtship and/or aggression ('activities'), and second, to enable large-scale genetic and circuit-perturbation screens. The system is simple and inexpensive to build and replicate, functions automatically and permits measurements of multiple fly pairs simultaneously. The application of this approach, together with appropriate multiplex aggression arena configurations[25] (**Supplementary Methods** online), should enable large-scale genetic and circuit-based screens of these behaviors.

RESULTS

Hardware and software

We used a double-arena adapted from a recently developed system[23] (**Fig. 1a**). A consumer camcorder, connected to a personal computer, filmed one pair of flies per arena. This setup can be adapted to a medium-throughput mode, consisting of four double arenas (**Fig. 1b–d** and **Supplementary Methods**).

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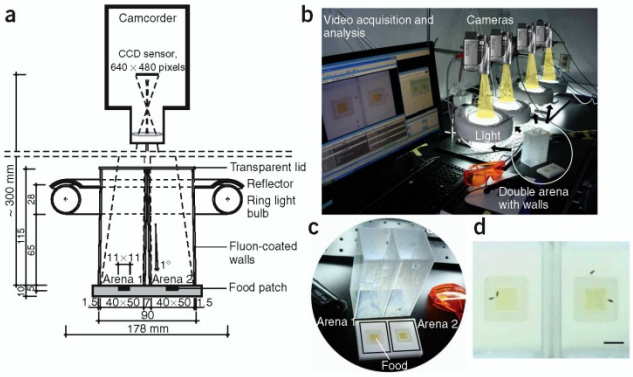

Figure 1 | Imaging setup for genetic screens in *Drosophila*. (**a**) Schematic of a lateral cut through our double chamber (all lengths in mm). CCD, charge-coupled device. (**b**) Example of a medium-throughput behavioral screening assay setup: 4 double chambers, 4 cameras, 2 personal computers and standard video-acquisition software. (**c**) Double chamber with the walls removed to expose the floor. (**d**) Camera view of the double chamber. Each of the two arenas has food in the center, surrounded by agarose; walls are coated with Fluon (Dupont fluoroproducts). Inner walls were tilted by 1°. Scale bar, 10 mm.

Our software (**Supplementary Software** online) consists of six modules: video import, ground truthing, calibration, fly detection and tracking, action detection and graphical user interface. We describe fly detection and tracking as well as action detection here and in **Figure 2**; for other modules, see **Supplementary Methods**.

Detection and tracking

The first step in tracking the flies is computing their silhouette (body and wings) (**Fig. 2a–c**). An important feature of our software is the ability to detect and measure the position of the fly's head and wings (**Fig. 2c,d**). We illustrate computing the orientation, θ, of a fly in **Figure 2e**. The bodies of abutting flies are resolved by fitting a two-component Gaussian mixture model[26] simultaneously to pixel location and brightness (**Fig. 2f** and **Supplementary Methods**).

At each video frame, 25 measurements (features) are computed, characterizing body size, wing pose, and position and velocity of the fly pair (**Fig. 2d,e,g** and **Supplementary Table 1** online). These measurements are the features used to detect actions.

In male-female assays the flies' identity is directly measurable as the female is larger. In male-male assays we painted a white dot on the back of one fly for identification. For unlabeled male-male pairs our software computes the most likely fly-specific trajectories (**Supplementary Methods**).

Action detection

The 25 measured features are used to detect fly actions. Lunging, tussling and wing threat are actions specific to aggression; wing extension (courtship 'song'), circling and copulation are specific to courtship; locomotion and chasing are common to both (**Fig. 3**).

A lunge is defined as one fly rearing briefly on its hind legs and snapping down onto the other fly[23] (**Fig. 3a**, **Supplementary Fig. 1a** online and **Supplementary Videos 1,2** online). Lunging is detected automatically by an example-based classifier in a two-step process (**Supplementary Methods**). First, probable lunges are selected among all frames by using ranges (intervals) on feature

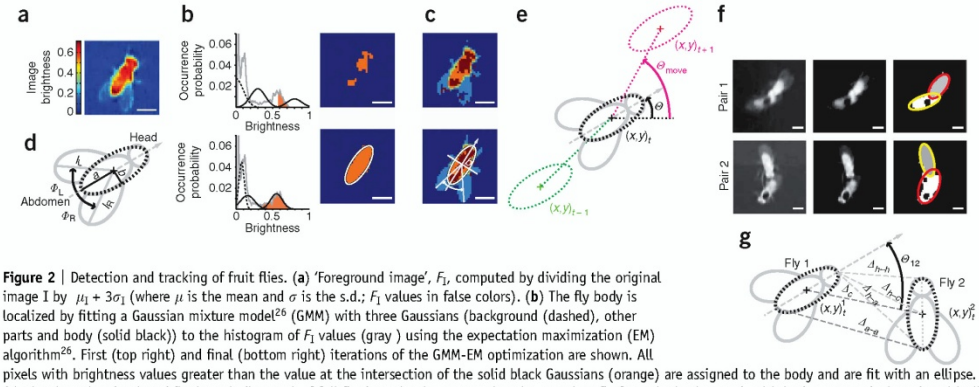

Figure 2 | Detection and tracking of fruit flies. (**a**) 'Foreground image', F_1, computed by dividing the original image I by $\mu_1 + 3\sigma_1$ (where μ is the mean and σ is the s.d.; F_1 values in false colors). (**b**) The fly body is localized by fitting a Gaussian mixture model[26] (GMM) with three Gaussians (background (dashed), other parts and body (solid black)) to the histogram of F_1 values (gray) using the expectation maximization (EM) algorithm[26]. First (top right) and final (bottom right) iterations of the GMM-EM optimization are shown. All pixels with brightness values greater than the value at the intersection of the solid black Gaussians (orange) are assigned to the body and are fit with an ellipse. (**c**) First iteration (top) and final result (bottom) of full fly detection by segmenting the complete fly from the background, with body parts and wings (empirically represented as four segments and colors)[30]. (**d**) Head and abdomen were resolved by dividing the fly along the minor axis *b* and comparing the brightness-value distribution of both halves (head is brighter; see **c**). The left and right wings (wing length l_L and l_R; and wing angle Φ_L and Φ_R) were measured by detecting, on each side of the fly's posterior half, the pixel with the furthest distance from the center of the ellipse (wing tip) in the segmented full fly. (**e**) Definition of fly orientation Θ and moving direction Θ_{move} (t, time). (**f**) Separation of occluding (pair 1) and touching flies (pair 2). Original image (left), foreground image (center) and final segmentation result with corresponding ellipses (right) are shown. (**g**) Definitions of additional features. Θ_{12}, angle between center of ellipse and head of fly 1 and center of ellipse of fly 2; *a* abdomen; *h*, head; *c*, center of ellipse; Λ, distance. Scale bars, 1 mm.

Figure 3 | Detectable actions. (**a–d**) Single images of side and top views showing lunging (**a**), tussling (**b**), wing threat (**c**) and copulation (**d**). (**e**,**f**) Sequential images showing wing extension and circling (**e**) and chasing (**f**). Scale bars, 1 mm. Times shown are relative to the first frame in each movie.

values as listed in **Supplementary Table 2** online. Second, probable lunges are accepted or rejected by a *k*-nearest-neighbor classifier[26] using 10 features (**Supplementary Table 3** online). The training examples comprised ~250 distinct expert-selected lunge events occurring in 20 min of recorded video for each of 8 fly pairs and a comparable number of negative examples.

We evaluated the performance of our lunge detector on a 20-min movie containing 139 lunges. We obtained ground truth (the accurate identification of all lunges in a movie) in two steps (see Methods and **Supplementary Methods**). We compared the algorithm's performance, as well as a second expert's annotations, to ground truth. The receiver operating characteristic[26] representing the fraction of false negatives (lunges present in the ground truth, but not detected) versus the number of false positives (detected, but not present in the ground truth) is shown, as the number of lunge-labeled nearest neighbors that are necessary to declare a lunge is varied (**Fig. 4a**). The threshold we selected for labeling a lunge yielded a detection rate of ~91%, representing 126 correct detections, with 13 false negatives and 7 false positives. Lowering the detection threshold decreases the probability for false negatives and increases the number of false positives.

We applied the lunge detector to 56 additional fly-pair movies (**Fig. 4b**) and found excellent agreement with the ground truth, with a correlation of 0.99, a bias of 1.5 lunges and an s.e.m. of 0.40 lunges. Note that automatic counting is very close to ground truth both when there are many lunges and when there are few.

We could detect other aggressive and courtship actions (see below) by using only the first of the two steps in the example-based classifier algorithm described above. We empirically determined features and ranges for each action by expert analysis of movies containing sample actions. The *k*-nearest-neighbor classifier step was unnecessary in these cases. We evaluated the performance of our system in detecting the other aggressive and courtship actions using the same approach as in the case of lunging. For all of these behaviors we measured detection rates of 90–100% (**Table 1**).

In aggressive tussling, both flies grip each other with their front legs[13] (**Fig. 3b**, **Supplementary Fig. 1a** and **Supplementary Video 3** online). The bodies face each other so that their axes of symmetry are parallel (body alignment) and form a single line. Connected solidly in this configuration, they move about in jerks with high velocity and acceleration. To detect and classify aggressive tussling, we compared 8 features to data ranges listed in **Supplementary Table 4** online. When all features are within their empirically determined ranges and this configuration is maintained for 0.3 s or longer, we flagged an aggressive tussling event.

Wing threat is characterized by a lateral extension of both wings by 80–90° followed by their elevation to a vertical extension of ~40° (**Fig. 3c**, **Supplementary Fig. 1a** and **Supplementary Videos 4,5** online). We observed both rapid, transient elevation of the wings as well as longer-lasting ($\geq$ 0.3 s) occurrences; the latter are typically, but not always, associated with a reduction in walking speed (velocity $\leq$ 5 mm s^{-1}). In this study, we restricted detection of wing threat to longer-lasting occurrences, as they are more easily discriminated from rapid wing flicking (see **Supplementary Table 5** online for features and ranges).

During courtship, males extend their wings laterally and vibrate them (~280 Hz) to produce a courtship 'song' (**Fig. 3e**, **Supplementary Fig. 1b** and **Supplementary Videos 6,7** online). Our system detects wing extension when the angle between the wing and the long axis of the fly body is greater than 60°, the fly is not standing up (fly length, as viewed from the camera, is maximal), and this configuration is maintained for 1 s or longer (see **Supplementary Table 6** online for features and ranges).

Circling is part of courtship and is detected when one fly drifts sideways in a circle with approximately constant velocity around the other fly (**Fig. 3e**, **Supplementary Fig. 1b** and **Supplementary Videos 6,7**; see **Supplementary Table 7** online for features and ranges).

Copulation involves a male fly approaching and mounting a female fly (**Fig. 3d**, **Supplementary Fig. 1b** and **Supplementary Videos 8,9** online). The beginning and end of copulation are characterized by an abrupt change in the distance between the two flies. During copulation their movements become coupled, and locomotion is decreased. Thus, to detect both the starting and ending time-points of copulation, we computed the mean and s.d. of inter-fly distances, Δ_c, within a moving 250-frame (8.3 s) window. We defined the earliest frame when the criteria of mean distance < 2 mm and s.d. < 0.3 mm are simultaneously met as the 'copulation start', and the last such frame as the 'copulation end' (see **Supplementary Table 8** online for features and ranges).

Our system detects chasing when the change of the head-center distance, $\delta\Delta_{h-c}$, between both flies (**Fig. 2g**) is small, both flies have

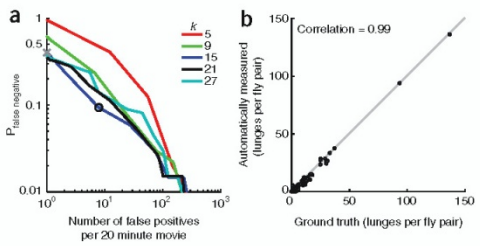

Figure 4 | Performance of action detection. (**a**) Performance of our lunge detector, described by the receiver operating characteristic. Each receiver operating characteristic curve gives the fraction of false-negatives (number of missed lunges divided by the total number of lunges on the ground truth) versus the number of false positives. Curves are shown for different values of k-nearest-neighbors constant k. Best performance was achieved for $k = 15$. The operating point of the system is shown by a black circle; it was obtained by labeling an action 'lunge' when 12 or more of its $k = 15$ nearest-neighbors were lunges. The performance of an expert human observer (40% missed lunges) is indicated by the ×. The expert detected 84 lunges, whereas our two-step process for establishing ground truth yielded 139 lunges. (**b**) Comparison between automatically measured lunges and the number of lunges in the ground truth for each of 56 20-min movies of fly pairs (dots). The gray line is the 45° reference.

the same, constant velocity, the distance between the flies is small but not zero, the chasing fly is oriented toward the chased fly, and the head of the chasing fly is behind the chased fly's abdomen (**Fig. 3f**, **Supplementary Fig. 1c** and **Supplementary Video 10** online). This configuration has to be maintained for 1 s or longer (see **Supplementary Table 9** online for features and ranges).

Genetic and environmental influences on social behavior

To validate the utility of our system for studying experimental perturbations of courtship and aggressive behavior, we first investigated whether it could detect previously described phenotypes produced by gene- or circuit-level manipulations. Octopamine is an insect biogenic amine, which is closely related to mammalian noradrenaline. It is critical in aggressive behavior in *Drosophila*[14]. A recent study showed that silencing of octopamine neurons decreases aggressive behavior[23]. We performed a similar manipulation, by expressing the inwardly rectifying potassium channel Kir2.1 (ref. 27) in tyrosine decarboxylase 2 (*Tdc2*)-expressing neurons, to suppress their electrical activity. *Tdc2-GAL4;UAS-Kir2.1* flies showed significant decreases in lunging ($P < 0.01$), tussling ($P < 0.05$) and wing-threats ($P < 0.01$) (**Fig. 5a–d** and **Supplementary Fig. 2a** and **3a**,**b** online). There was no significant change in chasing or total distance traveled ($P > 0.05$) (**Supplementary Fig. 2b**,**c**).

We also examined flies bearing a mutation in fruitless (*fru*), a sex-specifically spliced transcription factor that specifies gender-dimorphic fly behaviors[2,4]. Male fru^F flies, in which *fru* is spliced into a female-specific (inactive) form, exhibited a strong reduction in male-specific patterns of aggressive behavior (**Supplementary Fig. 4a–d** online), as previously reported[4].

We also studied the behavior of *Cha-GAL4;UAS-tra* (Cha-Tra) flies, in which all cholinergic neurons have been feminized owing to misexpression of the transformer gene[28]. Cha-Tra males exhibited little or no courtship activity toward females, but a robust increase in courtship toward other males (**Fig. 5e**,**f** and **Supplementary Fig. 2d**), at a frequency indistinguishable from wild-type Canton-S male-female pairs.

Cha-Tra male pairs also showed an increase in some aggressive behaviors compared to Canton-S male pairs (**Fig. 5g**,**h**, **Supplementary Fig. 2e** and **3c**,**d**), as previously reported (Y.B. Chan and E.A. Kravitz, *Cold Spring Harbor Laboratory Neurobiology of Drosophila Abstracts*, **42**, 2005). In addition, Cha-Tra male pairs had greater locomotor activity (**Supplementary Fig. 2f**). Lunging activity was significantly higher than in controls ($P < 0.001$), even after normalizing for distance traveled[23] ($P < 0.01$) (**Fig. 5i**). Wing threat was significantly lower in Cha-Tra flies ($P < 0.01$) (**Fig. 5j**); thus, not all aggressive actions were more frequent in Cha-Tra flies. Nevertheless, the total time spent in aggressive activity and chasing was significantly elevated in Cha-Tra males ($P < 0.01$ and $P < 0.001$, respectively; **Supplementary Fig. 2g**,**h**). Control experiments indicated that copulation could be detected in Canton-S male-female pairs (**Supplementary Fig. 2i**).

Our software allowed us to compute two-dimensional histograms showing the frequency of actions in each spatial location to detect phenotypes with an altered spatial distribution of behaviors (**Supplementary Fig. 5** online). The two-dimensional histograms revealed that Cha-Tra males performed a greater proportion of their tussling bouts on the central food patch, in comparison to controls (**Supplementary Fig. 5a**). Moreover, the pattern of Cha-Tra fly chasing was more intense around the perimeter of the arena, whereas that in controls was more uniformly distributed (**Supplementary Fig. 5b**).

We examined ethograms[13], which illustrate the frequency of each action, as well as the frequency with which one action was followed by the same or another action. We counted intervals >10 s without action as 'no action' nodes (data not shown). Both Canton-S and Cha-Tra males exhibited multiple transitions between courtship and

Table 1 | Performance evaluation of action detection

Behavior	Number of fly pairs	Number of events	Correct positives (%)	False negatives (%)	Number of false positives	Number of false positives per 20 minute movie	Number of false positives per event
Lunging[a,b]	1	139	90.7	9.3	7	7	0.05
Tussling	40	176	Not tested	Not tested	13	0.33	0.07
Wing threat[a,c]	40	87	94.3	5.7	4	0.1	0.04
Wing extension[d,e]	10	797	96.7	3.3	35	3.5	0.04
Circling[d,f]	10	422	99.8	0.2	18	1.8	0.04
Chasing[g]	6	400	98.0	2.0	4	0.67	0.01

[a]Wild-type Canton-S male-male fly pairs. [b]We tested 56 additional pairs, and the correlation with ground-truth data was 0.99. [c]We hand-counted 118 wing threats; 87/118 lasted longer than 0.3 s. False positives were ambiguous situations of wing threat or common wing extension. [d]Wild-type Canton-S male-female fly pairs. [e]We hand-counted 906 single wing extensions; 797/906 lasted longer than 1 s. False positives were due to segmentation errors. [f]Out of 435 hand-counted circling events, 422 had a minimum duration of 1 s. [g]*Cha-GAL4;UAS-tra* male-male fly pairs. Minimum duration, 1 s.

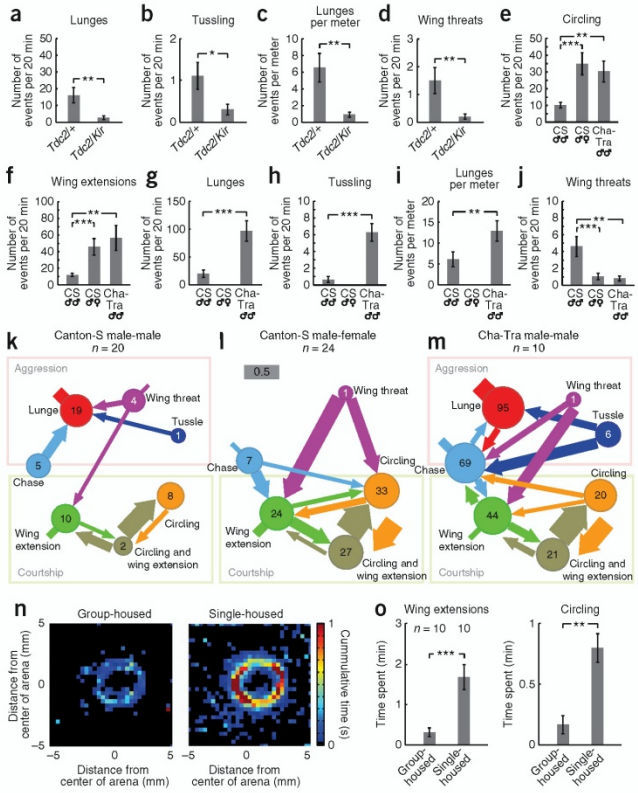

Figure 5 | Genetic and environmental influences on aggressive and courtship behavior. (**a–d**) Number of lunges (**a**), tussling events (**b**), lunges per meter (**c**) and wing threats (**d**) observed in octopamine control (*tdc2/+*, $n = 20$) and mutant (*tdc2/kir*, $n = 20$) flies. (**e–j**) Circling (**e**), wing extensions (**f**), lunges (**g**), tussling (**h**), lunges per meter (**i**) and wing threats (**j**) in Canton-S (CS) male-male ($n = 20$), male-female ($n = 24$) and Cha-Tra ($n = 10$) male-male pairs. Data represent mean ± s.e.m. (**k–m**) Ethograms, based on transition matrices for the indicated flies, showing transitions where the interval between a fly's action and the next action lasted ≤10 s. The transition probability is represented by the thickness of the arrows (normalized over all arrows that exit a node including the arrow into 'no action'). The gray bar represents a transition probability of 0.5. The arrow stumps represent the transition probability from one action into the same action. Circle diameters (logarithmically scaled) and numbers denote the average action frequencies. (**n**) Cumulative Canton-S male-fly positions while extending a wing toward a decapitated Canton-S female for group-housed flies (left; $n = 10$) and single-housed flies (right; $n = 10$). (**o**) Time spent performing wing extension and circling by single-housed and group-housed cs male-female pairs. Data represent mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

aggressive activities (**Fig. 5k–m**). Cha-Tra males additionally showed transitions between aggression and courtship and vice versa. They also showed an elevated amount of chasing activity. In Canton-S male pairs, chasing was followed most often by lunging, whereas in Cha-Tra males, chasing was followed with equal probability by either lunging, an aggressive action, or by wing extension, a courtship action (**Fig. 5k,m**). By contrast, in Canton-S male-female pairs, chasing by males was followed most often by wing extension (**Fig. 5l**). One interpretation is that in Canton-S male-male pairs, chasing is primarily an aggressive action, whereas in male-female pairs it is primarily a courtship action. In Cha-Tra male-male pairs, chasing may be indicative of either aggression or courtship, suggesting that these flies may have a deficit in gender recognition or discrimination. This observation is consistent with a recent study[29] reporting that feminization of octopaminergic and tyraminergic neurons by misexpression of Tra caused male-directed wing extensions to be followed primarily by male-male courtship.

Finally, to examine the effects of social experience, an environmental influence, on male-female courtship, we used our software to analyze wing extension and circling, two male-specific courtship actions. In this courtship assay the male was in the presence of an immobilized (decapitated) female in the center of the arena.

Previous studies have shown that raising post-eclosion *Drosophila* males in social isolation strongly increases aggressiveness, in comparison to flies raised in groups[24]. We observed that both wing extension and circling were significantly elevated in single-housed versus group-housed flies ($P < 0.001$ and $P < 0.01$, respectively) (**Fig. 5n,o**). Thus, social isolation increases both male-male aggressiveness and male-female courtship. This poses the question of whether these influences on social behavior are mediated by common or distinct mechanisms.

DISCUSSION

Our software detects wing postures, permitting measurements of wing threat and wing extension. Wing threat, in particular, is an interesting and important aggressive display because it is independent of locomotor activity. Indeed, we found that a genetic manipulation (Cha-Tra) that strongly increases lunging, tussling and chasing, decreased wing threat.

The time saved by our software is enormous. It takes us at least one hour to score manually one type of action in one 20-min fly-pair movie. To characterize aggression and courtship in a line of flies (all 8 actions in, for example, 12 fly pairs), one would require ~100 h of manual labor, as opposed to a few minutes to set up and run our system. As an example, the three ethograms presented in **Figure 5** would have taken ~270 person-hours to prepare. This capability affords the opportunity to compare multiple genotypes or wild-type genetic backgrounds, which would be virtually impossible to do manually.

The ability to monitor simultaneously both aggressive and courtship activities allows the computation of ethograms. This may prove to be valuable in determining whether aggression and courtship actions are part of a social behavior 'continuum' or whether these two activities represent discrete 'states' with 'state transitions' controlled by different circuits. Genetic or circuit-based screens can be performed to search for phenotypes that affect not only the ability to perform a particular action, but also that affect the probabilities of transition between actions.

Our system is completely automatic and self-calibrating; using it does not require special training, provided that the hardware setup is well reproduced. It has been designed for inexpensive implementation and easy replication. Fly behavior may be measured accurately in smaller arenas, allowing us to simultaneously monitor an array of arenas with each camera, thus permitting large-scale genetic screens with high throughput. Together, these features should open up aggression and courtship to powerful genetic screens. This in turn should help to illuminate the genes and neural circuits that control these important social behaviors and may reveal general principles of the organizational logic or control mechanisms that are evolutionarily conserved.

METHODS

Fly stocks and rearing conditions. Flies were reared in plastic vials containing standard fly medium (yeast, corn syrup and agar) at 25 °C, 60% humidity with a 12 h light-dark cycle. Newly eclosed males were single housed or group housed (10 flies per vial) for 4–6 days before we performed the behavioral assay. Virgin Canton-S females were collected shortly after eclosion and raised at 20 flies per vial for 4–6 d before we performed the courtship assay. *Cha-GAL4;UAS-tra* flies were made by crossing male *UAS-transformer* to female *Cha-GAL4* flies. The fru^F mutant was recently generated[2]. *Tdc2-GAL4;UAS-Kir2.1* flies were made by crossing male *Tdc2-GAL4* flies to female *UAS-Kir2.1* flies.

Aggression assay. We introduced two males of the same age into the double-arena setup by gentle aspiration without anesthesia and immediately video-captured them for 20 min. The temperature and humidity of the apparatus were set to ~25 °C and ~40%, respectively.

Courtship assay. Two types of courtship assays were performed. The apparatus and environmental conditions were as used in the aggression assay. One male and a virgin female were introduced into the apparatus by gentle aspiration without anesthesia and immediately video-captured for 30 min (24 pairs) to cover the copulation period. For all other actions only the first 20 min were analyzed. In another assay the female was decapitated and placed in the center of the food patch and replaced every hour. After the male was introduced into the apparatus both flies were immediately video-captured for 10 min.

Training and ground-truth data. We collected a hand-annotated database of lunging, wing threat, chasing, wing extension and circling to train our software and to measure its performance. Data used to train the detectors were produced by a human observer without additional checks. Data that were used for testing the system's performance were further processed to obtain a reliable 'ground truth'. Human experts tend to miss relevant events (in our observations, 30–40% of events are missed) for two reasons. (i) Different human observers use slightly different criteria, even when they agree on the overall action definition. (ii) A human observer's attention level changes over time during movie annotations. Therefore, to obtain reliable ground truth for performance testing, we devised an improved two-step procedure involving two experts (**Supplementary Methods**).

Statistical analyses. Kruskal-Wallis ANOVA was applied to detect overall differences among the unpaired groups. Significantly different groups were compared pairwise by the Mann-Whitney U-test. For all multiple comparisons, Bonferroni correction was applied.

Graphical user interfaces and software availability. Our software (**Supplementary Fig. 6a,b** and **Supplementary Software** online; software available as an executable file.) was run from graphical user interfaces allowing the user to visualize tracking and statistical data. Software source code is available free to academic and nonprofit investigators upon request. Commercial entities should contact the Caltech Office of Technology Transfer for licensing arrangements. Software updates will be available at http://vision.caltech.edu/cadabra/.

Additional methods. Additional information on the software, detection and tracking as well as method hardware is available in **Supplementary Methods**.

Note: Supplementary information is available on the Nature Methods website.

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