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Aggression and Courtship in *Drosophila*: Pheromonal Communication and Sex Recognition

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Abstract

Upon encountering a conspecific in the wild, males have to rapidly detect, integrate and process the most relevant signals to evoke an appropriate behavioral response. Courtship and aggression are the most important social behaviors in nature for procreation and survival: for males, making the right choice between the two depends on the ability to identify the sex of the other individual. In flies as in most species, males court females and attack other males. Although many sensory modalities are involved in sex recognition, chemosensory communication mediated by specific molecules that serve as pheromones plays a key role in helping males distinguish between courtship and aggression targets. The chemosensory signals used by flies include volatile and non-volatile compounds, detected by the olfactory and gustatory systems. Recently, several putative olfactory and gustatory receptors have been identified that play key roles in sex recognition, allowing investigators to begin to map the neuronal circuits that convey this sensory information to higher processing centers in the brain. Here, we describe how *Drosophila melanogaster* males use taste and smell to make correct behavioral choices.

Introduction

Understanding how the brain processes external stimuli to evoke behavior is a fundamental objective of neurobiology. On meeting a conspecific in the wild, animals must rapidly detect species-specific signals in a noisy environment and display an appropriate behavioral response. For males, the presence of a conspecific requires the ability to distinguish between potential mates and potential opponents. This in turn depends on the ability to determine the sex of the other animal. Throughout the animal kingdom, many different kinds of sensory signals are used for sex recognition, including visual displays, broadcast of olfactory signals, vocalizations, vibrations and use of unique surface molecules. The relative importance of the different signaling entities varies across species and this depends on specialized detectors utilized by the target organism. A commonly used mechanism in vertebrates and invertebrates is chemosensory communication. This is mediated by volatile and non-volatile pheromones that are released or releasable from the surface of one or both members of a signaling pair. Pheromones are chemical signals that convey messages to other members of the same species about sex, age, mating- and social-status and danger (Wyatt 2003). The first such compound identified was the sex pheromone *Bombykol* from the silk moth *Bombyx mori* (Butenandt et al. 1959). Since then, many molecules have been identified that serve as pheromones, and many of their mechanisms of action have been shown to be highly conserved (Law and Regnier 1971; Hildebrand 1995; Hildebrand and Shepherd 1997)). Pheromones elicit a specific reaction, for example a stereotyped behavioral response like courtship or aggression, but depending on the context, the same compound can have different effects. Pheromones can be distinguished from signature mixtures, which are variable subsets of molecules of an animal's chemical profile that are learnt by other animals of the same species, allowing them to distinguish among individuals (Wyatt 2010). Like most animals, fruit flies use taste and smell to detect chemical signals from their immediate environment. The chemosensory signals used include nonvolatile pheromones detected by

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the gustatory system and volatile pheromones detected by the olfactory system (Ebbs and Amrein 2007).

Like males of most species, *Drosophila* males court females and attack other males. Courtship and aggression are the stereotypical male-specific behaviors; these are complex, robust and innate behaviors that males routinely engage in, and both were described in *Drosophila* almost a century ago (Sturtevant 1915). The courtship ritual in *Drosophila* is a succession of fixed-action patterns that appear to involve different sensory modalities in its progression (Spieth 1974; Markow and Hanson 1981; Hall 1994; Greenspan and Ferveur 2000; Dickson 2008). The early steps involve mainly visual, vibratory and olfactory signals that allow males to find females and to orient their bodies towards them. A dance like circling around the front of females by males usually accompanies these initial steps. Next, males contact females by tapping their abdomens with their forelegs, a step that likely involves the male gustatory system in perception of non-volatile hydrocarbon pheromones on the female cuticle (Amrein and Thorne 2005; Ebbs and Amrein 2007). Then a series of additional maneuvers by males and females occur, linked by differing statistical likelihoods (Markow and Hanson 1981). The male will pursue a running-away female while vibrating one wing to perform a species-specific song. The female then usually slows down to allow the male to catch up and opens her vaginal plates while the male bends his abdomen to attempt copulation (Tompkins et al. 1982; Yu and Dickson 2008). Female choice is involved at the initiation of courtship and female rejection via a kicking behavior and/or a failure to open the vaginal plates can take place at any point during the ritual (Villella and Hall 2008). Roles for visual, gustatory and olfactory signals at different steps of the courtship ritual have been proposed (Crossley and Zuill 1970; Burnet and Connolly 1973; Venard 1980; Tompkins 1984; Stocker and Gendre 1989; Ferveur and Sureau 1996; Ferveur et al. 1997; Bray and Amrein 2003; Park et al. 2006; Kurtovic et al. 2007). More recently, studies have focused on whether multiple sensory modalities are involved in triggering the initiation of courtship, how these sensory modalities interact and whether a hierarchy exists in their patterns of usage (Ejima and Griffith 2008; Griffith and Ejima 2009; Krstic et al. 2009).

Unlike courtship, which is mainly directed by males towards females, both males and females show aggressive behavior towards individuals of their own sex and not towards the opposite sex (Dow and von Schilcher 1975; Jacobs 1978; Lee and Hall 2000; Chen et al. 2002). Agonistic interactions (commonly called bouts or fights) are composed of short meetings (or encounters) during which a variety of stereotypical behavioral patterns are displayed by pairs of same-sex flies (Hoffmann 1987; Chen et al. 2002). The behavioral patterns employed are sexually dimorphic (Nilsen et al. 2004). Fights between males start with low intensity patterns of agonistic behavior, particularly *fencing*. During each encounter, males touch each other repeatedly with their fore legs and labia. Similarly to the tapping step of the courtship ritual, this is likely an early instance of recognition that involves perception of cuticular hydrocarbons (CHs) via gustatory receptors located in the legs and mouth parts. After several encounters, the intensity of the behavioral patterns employed increases: the latency to start a fight in males is commonly defined as the latency to the first *lunge*. Lunging is the most distinctive and important male pattern of aggression: it is a direct attack in which an animal rises on its hind legs and snaps down rapidly and with considerable force on the opponent with its fore legs (Chen et al. 2002). It also is the predictor of the outcome of fights, with the first male to lunge, if the opponent runs away, being 16 times more likely to win the fight. Occasionally, fights between males escalate to higher intensity behavioral patterns like *boxing* and *tussling* (Chen et al. 2002). An exchange of agonistic patterns of behavior will continue until a dominance relationship is established with one male gaining control of the resources, after which the defeated animal retreats. Although often times the loser returns to the territory, he rarely lunges again (Yurkovic et al.

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2006; Miczek et al. 2007). The role of the different sensory modalities for the proper display of aggressive behavior is not as well resolved as in the case of courtship.

Both courtship and aggression are *plastic* behaviors influenced by the previous experience of an animal. They also are innate behaviors, and as such, their underlying circuitries are likely to have been pre-wired into the nervous system (Manoli et al. 2006; Dickson 2008). The genes of the sex determination cascade play key roles in building into the nervous system the potential for sex-specific behaviors (Billeter et al. 2006; Rideout et al. 2007; Pavlou and Goodwin 2012). In *Drosophila*, the sex of each cell is determined by the ratio of X chromosomes to autosomes via the gene *Sex lethal* (Sxl), that regulates splicing of the gene *transformer* (*tra*), resulting in expression of an active form of the splicing factor Tra only in females (Robinett et al. 2010). In turn, Tra regulates the expression of the transcription factors *fruitless* (*fru*) and *doublesex* (*dsx*) into male and female forms (Billeter et al. 2006). A prevailing view is that the main role of *dsx* is in the establishment of sexual dimorphism in morphology while *fru* is essential for sexual dimorphism in behavior, although considerable evidence now suggests a more complex scenario (Manoli et al. 2006; Kimura et al. 2008; Siwicki and Kravitz 2009; Pavlou and Goodwin 2012). Recently the Yamamoto and Dickson laboratories have identified a cluster of 20 male-specific Fru- expressing interneurons named *P1*, whose activation induces pulse song production as well as other elements of male courtship behavior (Koganezawa et al. 2009) even in the absence of a mating target (von Philipsborn et al. 2011; Kohatsu et al. 2011). The *P1* neuron cluster is located in the lateral protocerebrum, a higher brain center that has been involved in receiving sensory input from visual, auditory, olfactory and gustatory signals.

In this review, we ask how *Drosophila melanogaster* males use both olfactory and gustatory chemical signals to distinguish between aggression and courtship targets. In recent years, several putative pheromones and the olfactory and gustatory receptors involved in sex recognition have been identified. With the vast and versatile genetic tools available for research in fruit flies this has made it possible to begin to map the neuronal circuits through which pheromones concerned with social behavior reach higher order processing centers in the brain.

Sex recognition and male behavioral choice

When a male enters a territory and encounters a conspecific, a multiplicity of sensory cues are available that might provide the information required to tell the sex of the other animal in order to execute the appropriate behavioral response. The first signals perceived are likely to be visual cues, but little evidence is available that males discriminate between males and females solely based on morphological features. Auditory cues derived from wing and body movements might alert a male to the presence of a second fly, and thereby might arouse the intruder male (Ejima and Griffith 2008; Krstic et al. 2009). The prevailing view places chemosensory signals at the top of the hierarchy of sensory signals required for sex recognition. Olfactory signals would come first, as there are volatile and semi-volatile molecules that can act at a distance between flies and signal gender, while gustatory signals come into play once the animals are in physical contact with each other (Amrein and Thorne 2005; Touhara and Vosshall 2009). Detailed reviews of the anatomical and molecular structure of the specialized organs concerned with the chemosensory perceptions of taste and smell in flies already exist in the literature (Vosshall and Stocker 2007). Therefore only a superficial accounting of this information relating to social behavior will be presented below.

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Olfactory signaling

The *Drosophila* olfactory system involves two specialized appendages on fly heads, the antennae (mainly the third segment) and the maxillary palps (Stocker 1994). These appendages contain sensory hairs (sensilla of various types, with male female differences in number and distribution, see Stocker 1994), each of which contains between two and four olfactory receptor neurons (ORNs). Other modalities of sensilla and sensory neurons also are found in these locations. The olfactory receptor neurons project their axons centrally to the antennal lobes of the brain, which are divided into smaller units called glomeruli. The anatomical features of olfactory sensilla, olfactory receptor neurons and the antennal lobe and its glomeruli have been thoroughly described in the literature and will not be further described here (Stocker 1994; Hildebrand and Shepherd 1997). The family of olfactory receptors involves 60 genes that putatively encode for 62 predicted G protein-coupled receptors (Buck and Axel 1991; Mombaerts 1999; Galizia et al. 1999; Vosshall et al. 1999; Clyne et al. 1999; Gao and Chess 1999; Robertson et al. 2003). As in the mammalian system, expression studies and connectivity maps support the *one neuron-one receptor* and *one glomerulus-one receptor* models (Couto et al. 2005). In the antennae, olfactory receptors, housed in receptor neurons are found within three major morphologic types of sensilla: trichoid, basiconic and coeloconic. Of these, the trichoid sensilla do not respond strongly to food odors, but do respond well to fly odors (van der Goes van Naters and Carlson 2007). These are divided into 3 subcategories, T1, T2 and T3, which house one, two or three ORNs respectively, and represent about 20% of the sensilla on the antennal surface. By flowing odors of males and virgin females over the different categories of trichoid sensilla and recording electrophysiological responses, the Carlson laboratory demonstrated that T1 sensilla responded only to male odors, while T2 and T3 sensilla responded both to male and virgin female odors (van der Goes van Naters and Carlson 2007). These same authors then demonstrated using an “empty neuron technique” that 4 receptor subtypes, OR47b, OR 65a, OR67d and OR88a, responded to fly odors, but only two responded to cis-Vaccenyl acetate (cVa), a molecule transferred from males to females as a pheromone during copulation (see below).

The oxygenated hydrocarbon cVA has been known about for over 40 years, (Butterworth 1969). cVA is a semi-volatile compound that is stored and synthesized in the ejaculatory bulb of males (Butterworth 1969; Mane et al. 1983; Guiraudie-Capraz et al. 2007), and transferred to females during copulation where it reduces their attractiveness to males (Butterworth 1969; Brieger and Butterworth 1970; Jallon 1984; Ejima et al. 2007). Recently, it has been shown to have effects on both sexual and aggressive behavior (Kurtovic et al. 2007; Datta et al. 2008; Wang and Anderson 2010; Ronderos and Smith 2010). Its effects are sexually dimorphic, since it acts as an antiaphrodisiac for males (Zawistowski and Richmond 1986), while it enhances sexual receptivity in females (Kurtovic et al. 2007). Initially, cVA was described as an aggregation factor, attracting other flies to food sources (Bartelt et al. 1985). In this role it remains unclear whether it has an effect on its own or whether other compounds are involved, since different results were obtained by investigators using different experimental paradigms (Xu et al. 2005). The roles of cVA as a sex pheromone appear to be context-dependent, since it seems to have different functions depending on the social and physical environment (Bartelt et al. 1985; Schlief and Wilson 2007; Wang and Anderson 2010). Moreover, the inhibitory or excitatory nature of the actions of cVA might depend on the dose (Bartelt et al. 1985; van der Goes van Naters and Carlson 2007; Kurtovic et al. 2007). In this review, we will focus on the two olfactory receptors that have been reported to be involved in courtship and aggressive behavior, both of which mediate responses to cVA: Or67d, expressed in the T1 trichoid sensilla in the antennae, and Or65a which is expressed in the T3 trichoid sensilla (Fishilevich and Vosshall

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2005; Couto et al. 2005; Ha and Smith 2006). Two additional olfactory receptors have been reported to respond to fly odors, Or47b and Or88a (van der Goes van Naters and Carlson 2007).

Gustatory signaling

The gustatory receptor system is more widely distributed on fly surfaces than the olfactory system, with taste signals conveyed by sensory neurons present in gustatory organs distributed throughout the body. Labial palps at the end of the proboscis, internal mouthpart organs, distal forelegs and wing margins contain sensory structures (sensilla) called taste bristles and pegs, that usually contain 2–4 chemosensory and one mechanosensory neurons (Amrein 2004; Amrein and Thorne 2005). A sexual dimorphism exists on the forelegs where male flies have about a dozen additional receptors compared to females. The main gustatory processing centers in the brain are the subesophageal ganglion (SOG) and the tritocerebrum towards which gustatory-receptor neurons (GRNs) on the head and legs send their axons (Stocker and Schorderet 1981; Rajashekhar and Singh 1994; Thorne et al. 2004; Wang et al. 2004; Miyazaki and Ito 2010; Weiss et al. 2011). *Drosophila* gustatory receptors involve a family of sixty genes that putatively encode 68 different seven-transmembrane receptors (Clyne et al. 2000; Dunipace et al. 2001; Scott et al. 2001; Robertson et al. 2003). GRNs, in contrast to ORNs contain multiple rather than single receptors leading to suggestions of an as yet unexplained complex coding of taste including pheromone detection. No direct line, however, appears to link individual receptors with specific regions of target areas as in olfactory glomeruli, or with individual tastes. In a heroic effort gustatory receptor distributions and the response characteristics to an array of bitter tastants for all 31 taste hairs in the labellum was mapped, and it was revealed that a complex combinatorial code governs bitter information processing among gustatory sensilla in this primary sensory receiving area (Weiss et al. 2011). Here we will focus on two gustatory receptors, Gr68a, which has been reported to be required for an appropriate display of courtship behavior towards females, and Gr32a, which mediates detection of male HCs, having a role both in suppressing male-male courtship and in promoting aggression towards other males. Recently, it has been shown that loss of Gr39a also leads to a reduction in courtship towards females, suggesting that this receptor too may be involved in detection of female dienes (Watanabe et al. 2011). To date, the naturally occurring ligands for Gr68a and Gr39a remain to be identified.

Most of the compounds used by flies for sexual recognition are long chain hydrocarbons derived from fatty acids, made by epidermal cells called oenocytes and deposited on the fly's cuticular surface via a still poorly understood mechanism (Ferveur 1997; Jallon et al. 1997; Ferveur 2005; Krupp et al. 2008; Wicker-Thomas et al. 2009; Billeter et al. 2009). Large numbers of CHs are found on the surfaces of flies, along with other as yet not well characterized substances (e.g., oxygen containing hydrocarbons). All the CHs that have been identified as pheromones are unsaturated long-chain compounds, and some are sexually dimorphic: only females produce dienes, several of which induce male courtship, while males produce monoenes that have been shown to inhibit courtship from other males (Jallon 1984; Foley et al. 2007; Wicker-Thomas 2007; Arienti et al. 2010). Two desaturases are involved in CH production: the enzyme *Desat1,* is expressed in males and females, both in the oenocytes and in the fat body (Marcillac et al. 2005b; Marcillac et al. 2005a; Ueyama et al. 2005; Bousquet et al. 2009; Bousquet et al. 2012), and *DesatF*, is specifically expressed in female oenocytes (Chertemps et al. 2006). CHs not only play roles in inter-specific courtship, they also contribute to species isolation (Coyne et al. 1994; Savarit et al. 1999; Coyne et al. 1999; Marcillac et al. 2005c; Bontonou et al. 2012). Elimination of oenocytes by expression of the proapoptotic gene *hid* makes *D. melanogaster* females attractive to

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males from sibling species that would not normally court them (Chertemps et al. 2006; Billeter et al. 2009).

Courtship behavior and olfactory signals

Pheromonal signaling concerned with courtship via the olfactory system in *Drosophila* is currently focused on the pathway involving detection of cVA. The first olfactory receptor identified for detection of cVA was Or67d. This receptor protein does not act alone to trigger a response to cVA, however. Instead it functions in a complex formed by cVA and several proteins including the extracellular olfactory binding protein LUSH, a protein synthesized by non-neuronal support cells surrounding the receptor bearing neurons (Xu et al. 2005; Ha and Smith 2006). An activated form of LUSH can induce responses in sensory neurons even in the absence of cVA (Laughlin et al. 2008). Expressing Or67d in olfactory neurons that usually do not express the protein can make them responsive to cVA (Ha and Smith 2006), but an array of other proteins including the sensory neuron membrane protein (SNMP) and LUSH are necessary to confer close to normal levels of responsiveness (for an excellent review of the complexity of olfactory detection mechanisms for volatile compounds and the roles of ORNs in this process, see Ronderos and Smith 2009). A role for OR67d in the courtship-suppressing effects of cVA was based on the observation that Or67d mutant males show abnormally high levels of courtship toward males and mated females (Kurtovic et al. 2007). The same study and others showed that the effects of cVA as an antiaphrodisiac for females as well are mediated by this receptor. Or67d-expressing neurons innervate mainly a single antennal lobe glomerulus (DA1) (Kurtovic et al. 2007), and although sexually dimorphic in size (Stockinger and Dickson 2005, Kurtovic 2007), this glomerulus shows no differences in elevation of intracellular Ca^{2+} concentration or in electrophysiological responses to cVA between males and females (Datta et al. 2008). Using a photoactivable green fluorescent protein (PA-GFP), Datta *et al* (2008) investigated the circuitry mediating responses to cVA, and found that projection neurons from the DA1 glomerulus have sexually dimorphic axonal arbors in the lateral horn, a higher olfactory processing centre in the brain.

The receptor Or65 also can be activated by cVA, although the response is less pronounced than that of Or67d (Ha and Smith 2006; van der Goes van Naters and Carlson 2007). The behavioral role of Or65d in mediating responses to cVA was established based on the observation that initial courtship of virgin females can be blocked by expression of tetanus toxin in Or65a neurons (Ejima et al. 2007). Detection of cVA in the context of a female pheromonal profile normally induces a generalized suppression of male courtship, since this compound is found in mated and therefore unreceptive females. The Griffith laboratory showed that naive males show lower levels of courtship toward mated females than toward virgins of the same age, and that this effect can be reproduced by adding cVA through a laced filter across a two-compartment courtship chamber. In this study, expression of the tetanus toxin light chain (TNT), which blocks synaptic transmission, in Or65a, but not in Or67d- expressing neurons, suppressed the ability of cVA to inhibit initial courtship.

A reduction observed in female attractiveness after mating cannot be explained solely by an anti-aphrodisiac effect of cVA, since the levels of cVA drop after 24 hours while females remain unattractive for several days. Recently, a second male-specific pheromone has been identified, the monoacetylated diol 3-O-acetyl-1,3-dihydroxyoctacosa-11,19-diene (CH503). This compound, together with novel long-chain oxygenated hydrocarbons, was found by analyzing cuticular lipids from individual intact flies using a method that couples ultraviolet laser desorption/ionization with time-of-flight mass spectrometry (Yew et al. 2009). CH503 is abundant in males, and like cVA, is found in the male genital plate and is transferred to

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females through copulation, acting as an antiaphrodisiac to other males. Unlike cVA, however, which disappears from the surfaces of females in less than 48 hours, CH503 persists on the cuticle of females for at least ten days since it is less volatile. At that point it is the only detectable difference between mated and virgin females. CH503 also inhibits male courtship when applied to female targets (Yew et al. 2009). To date, the receptor for this compound has not been identified, and it remains to be determined whether CH503 is an olfactory or a gustatory cue.

Role of olfaction in male aggression

In addition to its role as an antiaphrodisiac, cVA has been shown to facilitate aggression (Wang and Anderson 2010). Addition of synthetic cVA to fighting chambers increases aggression, measured as increases in lunging behavior in group reared pairs of males. This effect is mediated by Or67d expressing neurons since it can be blocked by silencing these neurons via ectopic expression of the inwardly rectifying potassium channel Kir2.1 (Wang and Anderson 2010). The observation that the effect also was eliminated in Or67d mutant males indicates a role for the receptor itself, and not only for receptor expressing neurons. Silencing of Or65a neurons did not impair the aggression promoting effects of synthetic cVA. Increasing activity of Or67d-, and not Or65a-expressing neurons by expressing the bacterially derived sodium channel NaChBac also led to an increase in the numbers of lunges. By adding cVA to a chamber containing six males the authors demonstrated their dispersal from the food source, an effect that was absent in males in which Or67d neurons had been silenced. This indicates a further possible role for cVA in controlling population density. While acute exposure to cVA appears to increase aggression, chronic exposure to this pheromone reduces male aggression, mimicking the effects of group housing (Liu et al. 2011). In flies as in other species, socially isolated individuals are more aggressive than those raised in groups, an effect that might result from prior fighting and courtship experience and already established hierarchical relationships in the group-housed animals (Yurkovic et al. 2006). It is of interest that inhibition of Or67d neuronal activity attenuated the effects on aggression of acute but not chronic exposure to cVA. In contrast, silencing of Or65a neurons both during chronic cVA exposure and in group housed animals prevented the reduction in aggression (Liu et al. 2011).

Among the genes that are differentially expressed in males raised in isolation or grouped are a number of genes expressed in chemosensory neurons. This raises the possibility that social context influences the sensitivity of sensory neurons to pheromones (Zhou et al. 2009). One possible candidate gene altered under these conditions codes for cryptochrome P450 (Cyp6a20). Up-regulation of levels of expression of this gene were observed by comparing gene expression profiles in males showing different levels of aggression (Dierick and Greenspan 2006) and when comparing single- and group-reared males (Wang et al. 2008). The pattern of expression of this gene overlaps with that of LUSH in the support cells of sensilla and antennae, although expression of this gene also is seen in some neurons within the brain. A possibility suggested by the latter authors is that Cyp6a20 somehow modulates the sensitivity of Or67d neurons to cVA.

Role of gustatory signaling in male courtship behavior

Gustatory receptor neurons allow male flies to sense CHs distributed on the cuticular surfaces of other flies they come in contact with (Amrein and Thorne 2005; Villella and Hall 2008; Wicker-Thomas et al. 2009; Billeter and Levine 2012). Drosophila CHs have very low volatility and therefore are believed to be perceived mostly by contact with gustatory hairs, although it has been hypothesized that flies can also detect them at short distances via the

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olfactory system. Touching conspecifics with the legs and mouthparts are seen early in interactions in both male-female and male-male encounters (Schneider et al. 2012). As mentioned above, the cuticular pheromonal profiles of *D. melanogaster* are sexually dimorphic with the most abundant compounds on female cuticles being 7,11-heptacosadiene (7,11-HD; 27:2) and 7,11-nonacosadiene (7,11-ND; 29:2), and on males being 7-tricosene (7-T; 23:1) and 7-pentacosene (7-P; 25:1) (Amrein 2004; Ebbs and Amrein 2007). The dienes present on females, especially 7,11-HD, have been demonstrated repeatedly to induce male courtship while the male-specific monoene, 7-T, acts as an antiaphrodisiac, inhibiting courtship from other males (Antony and Jallon 1982; Antony et al. 1985; Tram and Wolfner 1998).

Activation of male courtship towards females

The first gustatory receptor identified for the detection of female CHs was Gr68a (Bray and Amrein 2003), which is expressed in male forelegs and in a number of mechanosensory neurons in both the legs and the Johnston's organ (Ejima and Griffith 2008). Male flies in which Gr68a-expressing neurons are inactivated by expression of Tetanus Toxin (TNT), or where Gr68a levels are reduced using RNAi, do not proceed normally through the courtship ritual. Instead, they appear to get stuck at the tapping step, a phenotype that could be explained by an inability to detect female pheromones necessary to proceed to further steps (Bray and Amrein 2003). In a separate study, Ejima *et al* showed that males in which Gr68a neurons are silenced via tetanus toxin (TNT) displayed normal courtship towards immobilized (headless) females but impaired courtship towards intact females. Their results suggest that the observed courtship phenotype is not due solely to an impairment in gustatory signaling: instead they suggest a role for Gr68a-positive mechanosensory neurons in motion detection, which appears to be necessary for courtship initiation (Ejima and Griffith 2008).

Suppression of male courtship towards other males

Two gustatory receptors have been reported to sense male pheromonal signals that suppress courtship from other males, Gr32a and Gr33a (Miyamoto and Amrein 2008; Moon et al. 2009). Both receptors are co-expressed in neurons that express Gr66a and both are implicated in the detection of bitter compounds (Wang et al. 2004; Marella et al. 2006) and in sensing the male pheromone 7-T (Lacaille et al. 2007). The loss of Gr32a expression results in an increase in courtship towards headless wild type males, while no effect is seen on the intensity of male courtship towards females (Miyamoto and Amrein 2008). A similar result is obtained by elimination of all Gr32a expressing neurons (Miyamoto and Amrein 2008). However, this mutation has no effect on courtship behavior towards intact males, suggesting that the inability to detect antiaphrodisiacs caused by the Gr32a mutation is not sufficient to increase courtship towards males in the presence of other behavioral signals that act as rejection cues (Wang et al. 2011).

When oenocytes are eliminated in male flies by expression of the proapoptotic gene *hid*, wild type males court these males at high levels (Billeter et al. 2009). Recently, it has been shown that perfuming oenocyteless, "blank" males with 7-T is sufficient to reduce courtship from wild type males to normal levels (Wang et al. 2011). This effect is not seen when 7-P is used and appears to be mediated by Gr32a, since in the absence of the receptor, males still court 7-T perfumed males at high levels (Wang et al. 2011). Many *Gr32a*-expressing neurons in the legs send their projections towards the suboesophageal ganglion (Koganezawa et al. 2010; Nojima et al. 2010). Some of these projections are in close proximity to the arborizations of a sexually dimorphic subpopulation of *fru* neurons, *fru*-

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mAL. Interestingly, the projections from *fru*-mAL neurons extend to the area where P1 neuron dendrites ramify.

In addition to gustatory receptors, as in the case of olfactory receptor neurons, other types of proteins have been identified as key components involved in the response to CHs. *CheB42* is a member of a family of secreted proteins expressed in sheath cells surrounding Gr68a-expressing neurons (Xu et al. 2002; Park et al. 2006). Mutating the *CheB42a* gene in males makes them progress through the courtship sequence faster than controls (Park et al. 2006). The *CheB42a* mutation alters the electrophysiological response of *pick-pocket25* (ppk25), a member of the degenerin/epithelial Na^+ channel (DEG/ENaC) family of sodium channels (Ben-Shahar et al. 2010). Mutations in the *ppk25* gene in males causes a reduction in courtship towards females but only in the dark (Lin et al. 2005). Moreover, both a knockdown of *ppk25* expression in gustatory neurons and synaptic inactivation of *ppk25*-expressing neurons impair male courtship (Starostina et al. 2012). In addition to *ppk25*, two other DEG/ENaC channels, *Ppk23* and *Ppk29,* have been shown to function in gustatory perception of contact pheromones (Pikielny 2012). Activation of neurons expressing *ppk23* enhances male courtship towards females and inhibits courtship towards males, but this response is seen only when visual and auditory signals are present (Toda et al. 2012; Thistle et al. 2012; Lu et al. 2012; Pikielny 2012). Moreover, *Ppk23*- expressing neurons can be divided based on their response to CHs, since one subpopulation responds to male monoenes and another sub population responds exclusively to female dienes (Thistle et al. 2012).

Gustatory signaling in male aggression

As described above, several lines of evidence suggest that contact pheromones detected by the gustatory system play a crucial role in deciding *what to do next* when a male encounters a conspecific (Billeter and Levine 2012). Males court females almost immediately upon contact, but it takes several encounters between two males until aggressive behavior is initiated. The presence of female pheromones is sufficient to trigger courtship behavior, since males that exhibit a feminized pheromonal profile elicit vigorous courtship behavior from wild type males (Ferveur et al. 1997).

In a previous study from our laboratory we masculinized the pheromone profile of females by expressing the RNAi for *transformer* in the oenocytes in order to test whether the presence of male pheromones is sufficient to trigger aggressive behavior (Fernandez et al. 2010). Such females (*oeno*$^{\text{traIR}}$) display high levels of monoenes and low levels of dienes on their cuticular surfaces, thereby resembling the CH profile found on males. Half of the males that were paired with *oeno*$^{\text{traIR}}$ females lunged at them, indicating that the presence of male HCs is sufficient to elicit aggression towards a target that is female in all other features. Surprisingly, males also attack females that exhibit normal female pheromonal profiles but that display male-like patterns of behavior. In these females, *tra* RNAi was expressed in the entire nervous system, generating females (*elav*$^{\text{traIR}}$) that display male patterns of behavior like singing or lunging. When both pheromones and behavior were switched, the normal response of males to both males and females could be reversed: males court rather than fight males that display a feminized pheromonal profile and feminized behavior, and fight rather than court when the female oenocytes and nervous system were masculinized.

This indicates that the presence of a female pheromonal profile is not sufficient to inhibit male aggression, since males attack not only males that display feminized CH profiles but also attack aggressive females that exhibit normal female pheromonal profiles. As expected, wild type males do not attack oenocyte-less, “blank” females, since they exhibit neither a male pheromonal profile nor male patterns of behavior. However, males still attack *oe*-

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males, although with reduced intensity, as these animals display normal patterns of aggression and synthesize cVA. Although acute exposure to cVA has been shown to facilitate aggression (Wang and Anderson 2010; Liu et al. 2011), it remains to be determined whether cVA is sufficient as a trigger for male aggression. The fact that males attack females who do not synthesize cVA, even before copulation when cVA would be transferred by males to females, suggests that cVA is not necessary for male aggression.

When *oe*- males are perfumed with male extracts, aggression levels elicited from wild type males are restored (Wang et al. 2011). In these experiments, *oe*- males were group housed, which reduces their aggressiveness compared to males kept in isolation. In a reciprocal manner to what was observed for suppression of courtship towards *oe*- males, perfuming them with 7-T, and not 7-P, restores normal levels of aggression from tester, wild type males (Wang et al. 2011). Males carrying a null mutation in the *Gr32* gene fail to display normal levels of aggression towards *oe*- males perfumed with 7-T, indicating that this receptor mediates the effects of this male pheromone both in suppressing courtship and in promoting aggression towards other males. Interestingly, exposure to cVA does not increase aggression in Gr32a mutant males, suggesting that a hierarchical relationship exists between the ways that 7-T and cVA regulate aggression.

Discussion

Social interactions rely on the ability of an individual to categorize other animals from the same species. Courtship towards females and aggression towards other males are the most important social behaviors displayed by *D. melanogaster* males, and both are strongly dependent on pheromonal communication mediated by chemosensory signals that are detected by the olfactory and gustatory systems, one functioning at a distance between flies the other requiring contact. An enormous literature describes these two systems in fruit flies. Despite massive efforts, however, many fundamental principles are missing of how olfactory and gustatory information is perceived, and subsequently parsed out in the nervous system to provide the information needed for rapid identification of a conspecific. Much excitement about how chemosensory information was transformed to electrical signals by sensory neurons, resulted from the discovery at about the millennium of two sets of approximately 60 member seven-transmembrane gene families; one for olfaction (ORs; (Touhara and Vosshall 2009)) and a second for gustation (GRs; (Montell 2009)). Moreover the finding that most olfactory sensory neurons contained a single OR and that these targeted single glomeruli in the primary olfactory region of the brain (the antennal lobe; (Ronderos and Smith 2009)), as in vertebrates, generated further hope that not only would olfaction be solved, but with the powerful genetic methods available in fruit flies, the work with flies would rapidly move ahead of the vertebrate studies. While in some areas concerned with the processing of information in the antennal lobe, this has turned out to be true (Wilson 2008; Masse et al. 2009), it was not until very recently that a detailed understanding of how ORs work along with multiple associated ancillary proteins to generate signals (Ronderos and Smith 2009). How GRs function is still not understood at that level of detail. A special problem with insect taste sensory neurons is that single neurons contain multiple GRs (up to 6 or more). In addition, taste sensory sensilla contain two to four distinct sensory neurons of this type along with mechanosensory neurons as well. Moreover, it could be that the neurons that express a particular OR or GR are themselves heterogeneous in terms of their response properties towards different tastants or pheromones depending on the complement of other receptors and ancillary proteins (binding proteins, etc.) immediately available to them within individual sensilla. It is not clear how such a complex arrangement at the sensory detection end, will yield the specific behavioral responses observed by manipulation of ORs and GRs. A special problem faced by insect

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sensory systems may be the prominence of importance of the detection of pheromones, which sometimes can be detected by target organisms at a single molecule level. Perhaps the specificity of behavioral responses resides in the circuitry surrounding the second order neurons within the primary sensory receiving areas of the brain. But how should one map the sensory neurons to their target areas within the primary response areas, if the receptor bearing neurons themselves associated with particular receptor subtypes respond to different ligands in a heterogeneous manner?

It also is clear that there are many unknown ligands that have been yet to be discovered for the large variety of ORs and GRs that are present. One possible example is CH503 (Yew et al. 2009), a long chain diol ester that was recently isolated from the genital region of male flies. Like cVA it is made only in males and transferred to females during copulation, but unlike cVA it lasts many days longer on the surfaces of female flies, possibly explaining the long-term period when male flies showed reduced attraction to females. With the complexity of fly surfaces beyond CHs, newer methods of detecting and analyzing fly surfaces (Yew et al. 2008; Yew et al. 2009; Everaerts et al. 2010) and the complicated world that surrounds flies, there will likely be many more natural substances found that may serve as ligands for remaining unidentified receptors. There also have been substances other than cVA and 7-T that have been identified as influencing the social behavior of flies, like 7-pentacosene, 7-heptacosene (7-H; 27:1) (Antony et al. 1985) and 9-pentacosene (9-P) (Ferveur and Sureau 1996; Siwicki et al. 2005). Also 5-tricosene (5-T) has been shown to inhibit male courtship towards other ales, and no particular receptors or mechanisms of action have been proposed for these substances to date.

Finally, in this article we survey a literature showing that levels of both courtship and aggression can be influenced by a relatively small number of chemical substances or by activation of their putative receptors. Such results demonstrate that these substances can *facilitate* or even be *sufficient* to turn on the behaviors. However, to date none of the compounds examined have been shown to be *necessary* to activate or suppress these behaviors. Moreover, the fact that males attack females that display normal female pheromonal profiles but utilize male patterns of aggression indicates that other senses can substitute for chemosensation in recognizing another fly as a competitor or a mate. Although there appears to be a hierarchy in the relative importance of different sensory modalities, redundancy is to be expected, as essential life and species-survival decisions are being made by individual animals in these meetings.

Acknowledgments

It's a pleasure for us to write this review to honor John Hildebrand on the celebration of his 70th birthday. John is a particularly close friend of one of us (EAK), beginning with his post-doctoral days in the Kravitz lab at Harvard Medical School from 1969–1972, and continuing to the present day. As a key Internet node on the "joke network", John lightens the daily woes of his many friends all over the world. He is a true biologist, a rare scientific find in the ever over-specialized neurobiology community, and I credit him with being the key person in my metamorphosis into a neuroethologist. We raise our glasses to John, a multiply honored scientist, a leader in the field of insect neuroethology, a responsible citizen of the greater scientific community, a champion of opening access to minorities for scientific careers, and a leader in neuroscience education throughout the world. Here is a small contribution dedicated to John about our foray into the world of animal chemical communication.

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