collection process until the trap is empty. A filtered aspirator (for example, Bioquip item #1135A) can be used through the slit to selectively remove specimens.

Using a sleeve expedites the collection process. Hollow cardboard mailing tubes (Uline model # S-10723) with plastic end caps (Uline model # S-7020) can be used as a sleeve (Figure 3a). Using a rotary tool, drill, or sharp blade, make a hole into the plastic cap of the tube so that the culture vial can tightly fit through (Figure 3b). Next, cut the tube to roughly the height of the bottle without the shell vial attached (Figure 3c). When collecting, slide the tube over the trap (Figure 3d). Specimens migrate upwards toward light into the culture vial (Figure 3e). Natural light or a lamp may be used.

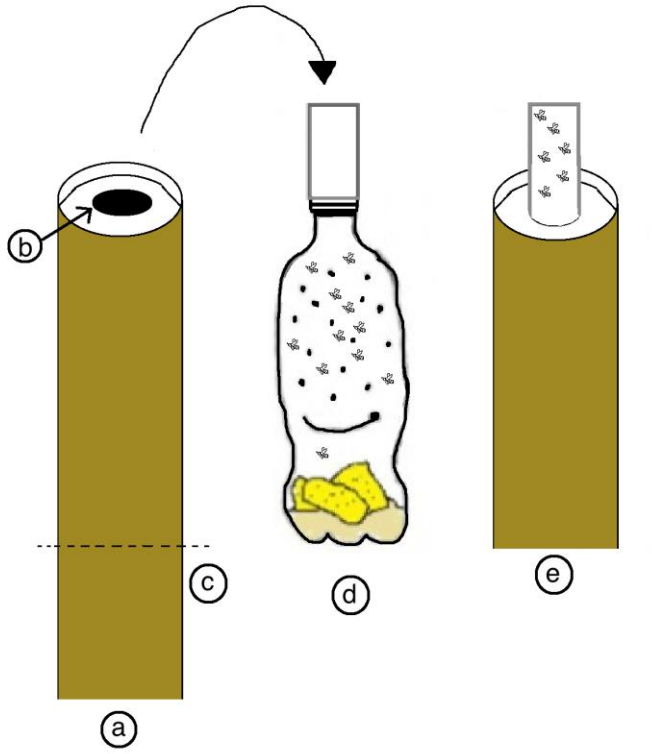

Figure 3. Collecting specimens using a sleeve.

A trap of this design is efficient because it is capable of trapping thousands of specimens 2 to 3 days after deployment, and it can be emptied quickly and easily. This trap has captured flies from many different species, including *D. melanogaster*, *D. simulans*, *D. busckii*, *D. robusta*, *D. affinis*, *D. tripunctata*, *D. immigrans*, *D. suzukii* (Freda and Braverman, 2013), and *Zaprionus indianus*.

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The impact of pheromones on sexual behavior in *D. melanogaster*: Recommendations for laboratory protocols.

Beck, Aaron P.[1,3], Erica F. Hamlin[2,3], Erika L. Hume[1,3], and Robert M. Hallock[1,4]. Skidmore College; [1]Neuroscience program, Skidmore College, 815 N Broadway, Saratoga Springs, NY 12866 [2]Psychology Department, Skidmore College, 815 N Broadway, Saratoga Springs, NY 12866; [3]These authors contributed equally to this work; [4]Corresponding author: rhallock@skidmore.edu

Abstract

Pheromones are conspecific chemical signals used throughout the animal kingdom that elicit behavioral responses in other organisms and are essential for intraspecies communication. *D.*

melanogaster utilize chemoreception for food acquisition, aggression, avoidance, courtship behavior, sexual recognition, and mate selection. Specific to sexual behavior, mating processes are mediated by volatile and non-volatile pheromone detection through olfactory and gustatory systems. These chemical signals are present in stock cultures housing *D. melanogaster* and can potentially influence behavior, leading to changes that may confound experimental data collection. Here, we review pheromonal processing and its role in sexual behavior, mating preferences, and courtship behavior; we illuminate specific pheromones and associated receptors and elucidate their involvement in each behavior, providing a thorough analysis of pheromone induced sexual behavior. Lastly, genetic manipulation and pheromonal application can be applied to regulate *D. melanogaster* behavior and enhance genetics research methodologies.
Keywords: Pheromones, *Drosophila*, *D. melanogaster*, fruit flies, laboratory protocols

Pheromones are conspecific chemical signals used throughout the animal kingdom that elicit behavioral responses in another organism. They are involved in behaviors such as trail marking, warning signals, territory marking, mating behavior, and sexual recognition (Wyatt, 2003). *Drosophila melanogaster* is a prominent animal model for genetics research, yet little attention is paid to the role of pheromones in its sexual behavior. *D. melanogaster* responds to many odorants critical for food acquisition, aggression, avoidance, courtship behavior, sexual recognition, and mate selection (Cande, Prud'homme, and Gompel, 2013; Dahanukar and Ray, 2011; Eastwood and Burnet, 1977; Fernandez and Kravitz, 2013; Herrero, 2012). These behaviors are mediated through synergistic cues from the olfactory and gustatory systems (Dickson, 2008; Sengupta, 2013; Smith, 2007; Wang and Anderson, 2013). In this review, we elaborate on how these chemoreception processes affect sexual behavior and their relevance to accurate and reliable experimental designs in the laboratory environment.

First, we discuss their pheromones and receptors that underlie pheromonal communication. Second, we describe courtship behaviors and mating preferences, and how specific pheromones and receptors can induce behavioral changes involved in mating. Next, we describe how current laboratory practices can be confounded with pheromones, and how laboratories could control for chemicals that stimulate chemosensory systems and subsequently affect aggression, courtship, and mating. Finally, we offer recommendations for laboratory protocols targeting the regulation of sexual behavior such as genetic manipulation and pheromonal application.

Pheromonal Detection and Processing

The olfactory and gustatory systems underlie pheromone detection in *D. melanogaster*. The detection of volatile pheromones occurs through the olfactory system, while nonvolatile pheromones are detected by the gustatory system (Dickson, 2008; Montell, 2009). The peripheral olfactory system includes the antennae and maxillary palps, which contain individual sensory neurons, called sensilla (Martin, Boto, Gomez-Diaz, and Alcorta, 2013). Within a sensillum are the cell bodies of the olfactory sensory neurons (OSNs) (See Figure 1). Protruding from each sensillum are smaller sensilla, which contain the dendrites of OSNs expressing multiple receptors. Figure 1B depicts a schematic of the dendritic and synaptic projections within each sensillum (Smith, 2007).

Several external areas of the body of *D. melanogaster*, including the labellum, pharynx, legs, and wing margins, contain gustatory receptor neurons (GRNs) that are expressed in separate sensilla (See Figure 2). Gustatory sensilla are more heavily distributed on the external structures such as the head, legs, and wing margins, and this allows for maximal chemosensory reception (Montell, 2009).

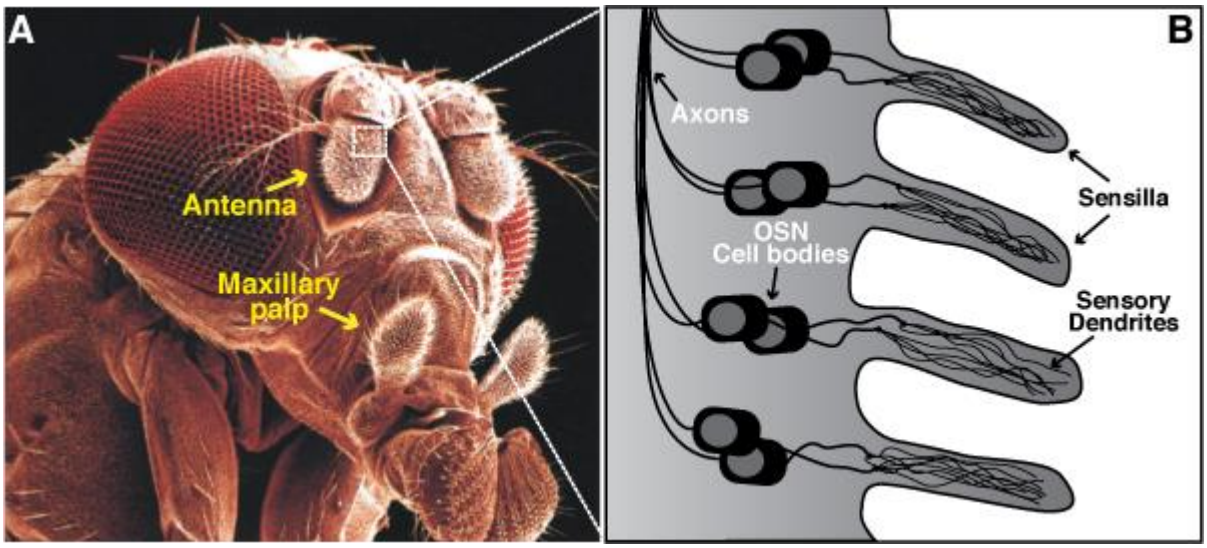

Figure 1. Peripheral olfactory system of *D. melanogaster*. A) Scanning electron image of antenna and maxillary palps. Photograph is courtesy of Juergen Berger, Max-Planck-Institute for Developmental Biology, Tuebingen. B) Schematic of olfactory sensory neurons (OSN) and their sensory dendrites. Schematic is courtesy of Walton Jones, Korea Advanced Institute of Science and Technology, South Korea.

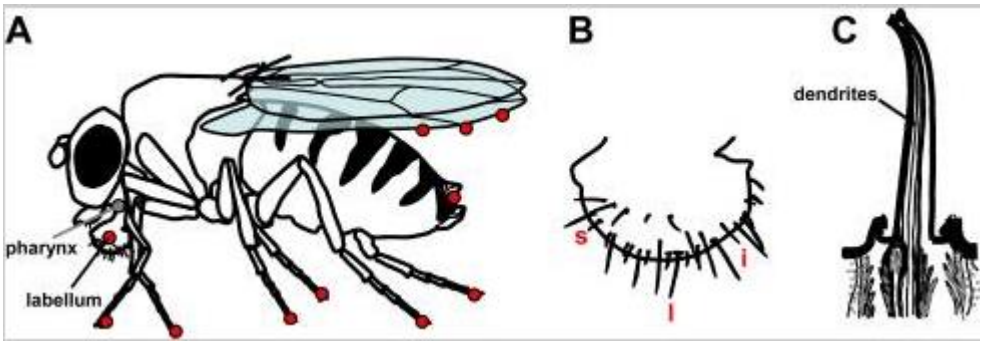

Figure 2. Gustatory system of *D. melanogaster*. A) Full body image of different regions of GRNs. grey dot - pharynx, red dots - (anterior-posterior) labellum, legs, wing margins. B) Labellum shown with protruding s-, l-, i-type sensilla. C) Sensillum with four dendrites extending to the terminal pore. From Montell (2009), reprinted with permission of Elsevier.

Further, this combination of olfactory and gustatory cues allows for a wide range of pheromonal detection.

The information from the peripheral olfactory and gustatory systems is then relayed to the central nervous system where neuroanatomical structures act as the processing centers in pheromonal signaling. Central structures in *D. melanogaster* include the suboesophageal ganglion (SG), antennal

lobes (analogue of the mammalian olfactory bulb), mushroom bodies, and the central complex (Heisenberg, 2003). Each brain region is essential for particular information processing. More specifically, the SG acts as the primary taste center of the central nervous system and receives information from the GRNs (Dickson, 2008). Antennal lobes integrate sensory information from the antennae and serve as downstream targets of OSN projections. The mushroom bodies are involved in odor perception and the memory induction of previous chemical signals, which can influence motor behavior (Crittenden *et al.*, 1998; Dickson, 2008; Heisenberg, 2003), and the central complex (CC) performs a global integration of related information.

Each neuroanatomical region can be broken down into more specialized regions known as glomeruli; OSNs and GRNs that express specific receptors target specific glomeruli (Dickson, 2008; Smith, 2007). Moreover, individual olfactory neurons mediate unique behaviors in response to sex pheromones (Kurtovic, 2007). This specialized chemotopic map aids in spatial and temporal orientation of a pheromone source, a critical mechanism for pheromonal detection and survival (Agarwal and Isacoff, 2011). Other neuronal circuits regulating aversion and attraction are also involved in processing and internalizing pheromonal information (Gao, 2013). This integrative neuronal circuitry thus underlies pheromonal processing and resulting behavior.

Pheromones and Receptors

The pheromones that *D. melanogaster* use to identify conspecifics and potential sexual partners are long-chain cuticular hydrocarbons (CHs) that are produced in the epidermal oenocytes of the abdomen (Krupp and Levine, 2010). Pheromones are composed of species-specific blends of these hydrocarbons and have evolved over many generations (Kent *et al.*, 2007; Billeter *et al.*, 2009). They can allow a male to recognize a conspecific, determine its sex, determine past courtship experience, and finally its receptivity (Billeter *et al.*, 2009; Ejima, 2012; Keleman *et al.*, 2012). The chemical composition of these hydrocarbons may vary in terms of chain length and bond position, including methyls, alkanes, fatty acids, and 5-, 7-, and 9-alkenes (Kent *et al.*, 2007).

Furthermore, the total abundance of each of these blends of compounds, distinguished by chemical composition, may have distinct rhythms of expression and activation over the course of both a 24-hour photoperiod and the individual's circadian free-running activity period (Chatterjee and Hardin, 2010; Kent *et al.*, 2007). The pigment-dispersing factor's (PDF) signaling pathway is a checkpoint in the pheromonal production cycle of the oenocytes, and disruption of this pathway can lead to sexually dimorphic differences in mating behavior (Krupp *et al.*, 2013). There are daily fluctuations of pheromone production in the laboratory setting that must be considered with respect to the time of day that data are recorded and collected.

Several pheromones are important in sexual behavior of *D. melanogaster*. 11-*cis*-vaccenyl acetate (cVA) is a non-volatile CH that is produced in the oenocytes of sexually mature males and is deposited onto females during courtship, and this allows other males to distinguish her as a previously mated individual (Bartelt *et al.*, 1985; Datta *et al.*, 2008; Keleman *et al.*, 2012). Therefore, cVA serves as an inhibitory signal to other males. Ablating the oenocytes in females results in the elimination of this sex-specific inhibitory signal through shorter courtship latency and stronger maintenance of courtship length, and courting by males of other *Drosophila* species. A separate CH alkene, known as 7,11-heptacosadiene (7,11-HD), also functions as a chemosensory signal of heterospecificity in *D. melanogaster* (Billeter, Atallah, Krupp, Millar, and Levine, 2009; Fan *et al.*, 2013). These and additional *D. melanogaster* sex pheromones are listed in Table 1.

Many receptors associated with the sex pheromones are localized to multiple areas in the central and peripheral nervous systems. The SG receives input from the GRNs during courtship,

including a key receptor, Gr68a, and relays these nonvolatile signals to the CC for higher order processing. Or67d and Or65a are two important receptors, among a larger family of OSNs, on the T1 and T3 trichoid sensilla of the antennae, respectively. Axons from the receptors project to the antennal lobe, where primary olfactory information is interpreted as a map of glomerular activation (Agarwal and Isacoff, 2011; Dickson, 2008). DA1 is one glomerulus that receives pheromonal input in the form of coincidence detectors on the antennae, by which the spatiotemporal features of odorants in the local environment are integrated and interpreted (Keene and Waddell, 2007; Perez-Orive *et al.*, 2004).

Table 1. Known pheromones and their associated functions and receptors.

Pheromone	Function	Associated receptor(s)	Reference
11-*cis*-vaccenyl acetate (cVA)	Secreted in male semen; inhibits other males from mating for ~10 days; promotes aggression in pairs of male flies	Or67d, Or65a, DA1	Bartelt et al. (1985); Xu et al. (2005)
z-7 tricosene	Inhibitory signal in males that prevents other males from mating; tastes bitter	Gr32a (?), Gr33a	Lacaille et al. (2007)
7,11 heptacosadiene	Slow down courtship; identifies same species mates; low levels cause hyperstimulated activity	*ppk23*(+) sensory neurons	Billeter et al. (2009); Toda et al. (2012)
7,11 nonacosadiene	Additional female aphrodisiac pheromone		Ferveur & Sureau (1996)
20-hydroxyecdysone	Modulates courtship behavior towards females	DmDopEcR (GPCR)	Abrieux et al. (2013)
ppk25	Stimulatory pheromone	Gr68a	Dahanukar & Ray (2011)

Arborization of the neurons in downstream targets of these glomeruli may be sexually dimorphic. The binding of certain receptors, often by the same pheromone, elicits distinct behavioral responses in males and females (Datta *et al.*, 2008; Dickson, 2008; Kurtovic *et al.*, 2007). For example, there is evidence that the mechanism regulating cVA binding responses is sexually dimorphic, such that it inhibits mating behavior in males and promotes it in females (Kurtovic *et al.*, 2007). This dimorphism in neural circuitry is regulated by the *fruitless* gene, which promotes the development of male-specific interneurons in downstream projections of the glomeruli (Kimura *et al.*, 2005).

DopR1 receptors, expressed in the mushroom bodies, bind signals from dopaminergic neurons that aid in memory formation and learning in the male (Keleman *et al.*, 2012). The mushroom bodies and the CC are two major structures in the *D. melanogaster* brain that regulate different mechanisms for sex-specificity and heterospecificity, respectively (Sakai and Kitamoto, 2006). For example, 7,11-HD functions as both a mating signal in males and as a conspecific identifier (Billeter *et al.*, 2009; Dahanukar and Ray, 2011; Fernandez and Kravitz, 2013).

Courtship Behaviors

Courtship behaviors in *D. melanogaster* are dictated by specific pheromones, their receptors, and subsequent activation through olfactory/pheromonal pathways. The initiation of courtship

involves visual, vibratory, and olfactory signals (Fernandez and Kravitz, 2013; Wicker-Thomas and Hamann, 2008). This allows the individual flies to spatially orient themselves to one another during the mating process and to determine which available conspecifics are optimal mates. To begin, the male will circle around the front of the female. He will then approach her and tap her abdomen with his forelegs, which contain the Gr68a receptors (Dahanukar and Ray, 2011; Fernandez and Kravitz, 2013).

Table 2. Known sex pheromone receptors and corresponding locations.

Receptor	Function	Associated pheromone / ligand	Location	Reference
Or47b	Conveys sensitivity to conspecific odorants	(in accordance with input from the VA1v glomerulus in females)		Vosshall et al. (2000)
Or65a	Required in males for suppressing courtship	cVA	T3 trichoid sensilla on antenna	Ejima et al. (2007); Dahanukar & Ray (2011)
Or67d	Promotes male-male aggression	cVA	T1 trichoid sensilla on antenna	Kurtovic et al. (2007); Dahanukar & Ray (2011)
Or83b	Common subunit in majority of odorant receptors	cVA (in accordance with receptor Or67d)		Wicher et al. (2008)
DmDopEcR	Modulates neuronal signaling in males	20-hydroxyecdysone	Corresponding proteins in mushroom bodies	Abrieux et al. (2013)
Gr32a	Binds male-specific inhibitory signals		Projections to ventrolateral protocerebrum	Miyamoto & Amrein (2008)
Gr33a	Binds male-specific inhibitory signals	(possibly) z-7 tricosene		Moon et al. (2009)
Gr36a	Bitter taste receptor	Inhibitory sex pheromone		Clyne et al. (2000); Weiss et al. (2011)
Gr68a	Induces regular courtship in males and females	ppk25	(In males) on forelegs, in gustatory neurons, auditory neurons in Johnston's organ	Bray & Amrein (2003)

These Gr68a receptors bind nonvolatile pheromones from the female during the mating ritual (Fernandez and Kravitz, 2013) and determine whether the female has any traces of previously deposited sex peptide or cVA, both of which deter the male from further courting. Sex peptide is bound tightly to the sperm of the male (Chapmann *et al.*, 2003; Dickson, 2008). This peptide induces certain female post-mating behaviors, such as the reluctance to mate again (Wigby and Chapmann, 2005). When females mate with mutant males that do not possess the sex peptide, they maintain their desire to continue to mate (Dickson, 2008). However, virgin females are the most likely candidate to accept courtship from males (Odeen and Morray, 2008), and are therefore the most desired targets. Additionally, *ppk25* has been identified as another potential ligand for the male

Gr68a receptor (Dahanukar and Ray, 2011; Fernandez and Kravitz, 2013), functioning as a modulator of the gustatory perception of contact pheromones (Pikielny, 2012).

If the female escapes, the male will pursue her while singing a species-specific song with his wings (Fernandez and Kravitz, 2013). The female may then slow down and open her vaginal plates for the male to commence copulation. If the male is rejected by the female, she will display kicking behavior and will not open her vaginal plates (Fernandez and Kravitz, 2013). The direct contact between the male forelegs and the female abdomen is an important moment when both the olfactory and gustatory systems are incorporated into the mating process.

In the case of successful copulation, cVA is transferred to the female in the male ejaculate and marks her as an already mated female (Dahanukar and Ray, 2011). Males that express Or67d or Or65a receptors receive this pheromonal signal, which acts as an anti-aphrodisiac. In addition to these receptors, LUSH, an odorant binding protein, is necessary to elicit responsiveness to cVA (Dahanukar and Ray, 2011; Fernandez and Kravitz, 2013; Kim *et al.*, 1998; Martin *et al.*, 2013). Additionally, cVA activity is represented in the DA1 glomeruli (Fernandez and Kravitz, 2013). This glomerulus is sexually dimorphic in size and is larger in males, although there is no difference in responsivity to cVA in males or females (Fernandez and Kravitz, 2013).

CH503, another cuticular hydrocarbon, is a pheromone similar to cVA in that it deters males from courting mated females (Dahanukar and Ray, 2011). CH503 has been detected on mated females directly after copulation and lasts 10 days after mating, indicating that it is less volatile than cVA (Dahanukar and Ray, 2011). CH503, along with receptor Or67d, has also been linked to male aggressive behavior (Dahanukar and Ray, 2011). Although CH503 is linked to male-male aggression, we do not yet know the specific quantity or concentration that will induce aggression. If a male *D. melanogaster* lacks the Or67d or Or65a receptors, it will not recognize that the female in question has already mated and is no longer susceptible to its advances. This male-specific olfactory mechanism is used to differentiate between mated and virgin female *D. melanogaster*. Naïve males will still attempt to initiate courtship with previously mated females, albeit with less enthusiasm than with virgin females (Keleman *et al.*, 2012).

An integral part of courtship behavior is a male's ability to decipher rejection from acceptance by females during courtship. The presence of cVA is not the most important factor in mate choice, but rather the previous experience with a particular female and whether the male experienced success or failure at copulating (Fernandez and Kravitz, 2013). Mating success is elevated, through enhancement of sensitivity to cVA, by learning to discriminate between mated females and virgin females.

The most important factor in male *D. melanogaster* learned mating behavior is success or lack of success in mating attempts. Keleman *et al.* (2012) showed that male mating behavior is conditioned by the failure to mate, not simply the rejection. When males were paired with mated females, they learned not to waste their energy or pheromones, while males that were paired with virgins learned to attempt mating with any female, because their advances were always accepted. Thus, males trained by mated females knew not to waste their energy on previously mated females that they encountered subsequently, while males trained with virgins made continuous futile attempts to mate with previously mated females.

Many studies have investigated male-male courtship behavior. Gr33a is a receptor that has been linked to male inhibitory signals with 7-tricosene as a possible ligand (Dahanukar and Ray, 2011; Fernandez and Kravitz, 2013). Additionally, the Gr32a receptor binds male-inhibitory signals, and these signals are then projected to the ventrolateral protocerebrum to induce rejection of male-male courtship (Dahanukar and Ray, 2011; Fernandez and Kravitz, 2013). While the Gr68a receptor helps to regulate normal courtship between males and females, it also works as a receptor for virgin female pheromones and to trigger courtship behavior in males (Dahanukar and Ray, 2011). Given

the range of behavioral responses that are affected as a result of these documented pheromones and their corresponding receptors, changes in commonly used methodologies can be approached.

Laboratory Protocols and Applications

Sexual behavior in male and female *D. melanogaster* may confound an experimental design. *D. melanogaster* exhibit a relatively short time frame from pupal eclosion to sexual maturation (approximately 8 hrs), and experimenters must be consistently vigilant over this time period to limit reproductive contamination and inbreeding. This is accomplished by monitoring the eclosion rate of new offspring in order to maintain generational distinction.

Many protocols require the experimenter to remove any remaining adults one week after making a new cross, given that the female can lay hundreds of eggs during this time. When the offspring begin to eclose from the pupae 2 to 4 days after the adults are removed, the virgin females must be removed from the rest of the offspring (provided that these offspring are to be crossed with genetically different strain). This step is critical in preventing inbreeding with males from the same strain, rather than the target strain. In such a case, it would be impossible to determine whether the offspring of the new cross, which typically arise in large quantities, are from the target cross strain (no contamination), or from the same cross from which the parents were reproduced (contamination). Other experiments require multiple repetitions of this process in a short amount of time. Using pheromones to prolong a period of less contact and attraction between males and females would, therefore, be a practical and non-invasive method of extending the interval of time after sexual maturation occurs when the flies typically begin to interact, in order to prevent contamination in these types of experiments.

Genetic manipulation of pheromone receptors may be a simple way to regulate sexual behavior in *D. melanogaster*. For example, an experimenter may limit sexual behavior by inducing a loss of function mutation to receptors such as Gr68a or *fruitless*. Additionally, one may reduce the frequency of mating rituals by inducing a gain of function mutation to Or65a. These are plausible techniques, but mutant strains could potentially crossbreed with an unwanted strain, in turn affecting sexual behavior and subsequent experiments. However, genetic manipulations may induce permanent changes and inhibit mating behaviors. Pheromonal applications, on the other hand, may provide temporary control over sexual behavior.

Utilizing the intact pheromonal system to regulate sexual behavior can also prove advantageous. CH503 and cVA both deter courtship behavior between males and females. Application of CH503 would deter female-male courtship for 10 days, providing temporary control of *D. melanogaster* sexual behavior (Dahanukar and Ray, 2011; Yew *et al.*, 2009). To induce regular courtship after the application of CH503, the pheromones 7-11HD or *ppk25* could be applied. This is relatively easy and cost effective, and can create a more controlled environment for genetics research. However, limitations of CH503 and cVA application include (1) effects on male-male aggression, (2) necessary reapplication of pheromones, and (3) long term effects of pheromonal application on typical mating behavior.

Conclusion

This review has illustrated the intricacies of the *D. melanogaster* olfactory and gustatory systems and their role in pheromonal reception, examined how pheromones influence sexual behavior, courtship behavior and mate preference, and compiled major known pheromones and

receptors and their specific mechanisms of action. Additionally, genetics research on *D. melanogaster* may benefit through the manipulation of sexual behavior by pheromonal application. This can be used to prolong the interval of time from pupal eclosion to the first mating experience, which is usually relatively short and renders stock cultures easily susceptible to contamination. Extended investigations of application dose levels and interactions between these chemicals would provide a better understanding of how to monitor fecundity levels and keep contamination minimal.

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Utilizing phospho-histone H3 labeling in the *Drosophila* larval central nervous system to generate parametrically testable mitotic index data sets.

Reubens, Michael*, Wayne A. Rummings, Jr.*, Lucas T. Hopkins, and Tim W. Christensen. Department of Biology, East Carolina University, Greenville, NC 27858; Correspondence: christensent@ecu.edu. *These authors contributed equally to this work.

Introduction

In *Drosophila*, the central nervous system (CNS) of the third instar wandering larvae continues to be a highly utilized tissue for cytological studies investigating the mitotic phenotypes of mutations thought to impact cell-cycle progression and/or chromosome dynamics. The larval CNS is unique in that, unlike most other larval organs, it persists into the adult stage (Truman, 1990), and it is comprised of two major cell types all undergoing canonical cell cycles: the neuroblasts and the ganglion mother cells (Hofbauer and Campos-Ortega, 1990). There are many published protocols available in the literature for squashing and labeling larval CNS tissue for the generation of mitotic indices in an effort to characterize mitotic defects (Gatti, 1974; Gatti and Goldberg, 1991; Bently, 2001; Williams, 1992; Bolkan, 2007; Ayeni, 2013). However, in our experience many previously described methods, while useful for analysis of chromosome morphology, did not generate data sets that were amendable to parametric statistical testing such as t-tests and ANOVAs.

In the following report, we describe a variation of our existing protocol (Apger, 2010) for examining the progression of the canonical cell cycle of the larval CNS that utilizes the sensitivity of phospho-histone H3 (PH3) labeling to generate mitotic indices. The added sensitivity of the PH3 labeling method allows for the generation of data that satisfies the assumptions of parametric statistical testing. With these assumptions satisfied, statistical tests, such as t-test and ANOVAs, can now legitimately be used to compare the effects of different mutant alleles on cell-cycle progression.

Materials and Methods

Drosophila Stocks

The w^{1118} line was obtained from the Bloomington Stock Center (Flybase ID: FBst0006326). Flies were maintained at 25°C on *Drosophila* Diet Medium K12 (US Biological Cat #D9600-07B).

Tissue Acquisition

Wandering third instar larvae were collected and placed in a 16 well dissecting dish containing 100 µl of 1× PBS (140 mM NaCl, 2.7 mM KCl, 1.4 mM Na_2HPO_4, 1.8 mM KH_2PO_4).