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CHEMICAL CUES THAT GUIDE FEMALE REPRODUCTION IN *DROSOPHILA MELANOGASTER*

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Abstract

Chemicals released into the environment by food, predators and conspecifics play critical roles in *Drosophila* reproduction. Females and males live in an environment full of smells, whose molecules communicate to them the availability of food, potential mates, competitors or predators. Volatile chemicals derived from fruit, yeast growing on the fruit, and flies already present on the fruit attract *Drosophila*, concentrating flies at food sites, where they will also mate. Species-specific cuticular hydrocarbons displayed on female *Drosophila* as they mature are sensed by males and act as pheromones to stimulate mating by conspecific males and inhibit heterospecific mating. The pheromonal profile of a female is also responsive to her nutritional environment, providing an honest signal of her fertility potential. After mating, cuticular and semen hydrocarbons transferred by the male change the female's chemical profile. These molecules make the female less attractive to other males, thus protecting her mate's sperm investment. Females have evolved the capacity to counteract this inhibition by ejecting the semen hydrocarbon (along with the rest of the remaining ejaculate) a few hours after mating. Although this ejection can temporarily restore the female's attractiveness, shortly thereafter another male pheromone, a seminal peptide, decreases the female's propensity to re-mate, thus continuing to protect the male's investment. Females use olfaction and taste sensing to select optimal egg-laying sites, integrating cues for the availability of food for her offspring, and the presence of other flies and of harmful species. We argue that taking into account evolutionary considerations such as sexual conflict, and the ecological conditions in which flies live, is helpful in understanding the role of highly species-specific pheromones and blends thereof, as well as an individual's response to the chemical cues in its environment.

Keywords

Reproduction; Pheromones; Chemical cues; *Drosophila*; Seminal fluid proteins; Olfaction; Gustation

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INTRODUCTION

Sexual reproduction requires the interaction of a male and a female in order for their gametes to meet and combine resulting in the production of a zygote. The female typically exerts pre-copulatory selection on males based on a male's display of traits that are ideally honest indicators of his fecundity and his possession of alleles that confer adaptation to the environment. The female and her chosen mate then cooperate in the production of offspring. This simple picture omits significant, but less known mechanisms that impact the evolution and regulation of reproductive behaviors. First, females may "tune" their sexual receptivity to ecological conditions, and not solely to male quality. This is because the production of energy-intensive eggs by females is constrained by the available nutritional resources. The ecological community also matters, as the presence of predators and competing species can impact female reproductive decisions, such as how to recognize males from her own species, where to lay eggs or raise offspring (in taxa with parental care). Second, reproduction is a social phenomenon beyond the reproducing pair: finding a mate is easier in a group than when alone, risk of predation is reduced when in a group of conspecifics, and resource use by the offspring is facilitated by the presence of others. Thus, to maximize their reproductive output, females must not only judge male quality, but also integrate ecological conditions such as nutritional resources, presence of competing species, and social environment.

While reproduction is a cooperative process between males and females and between individual group members, it should also be viewed through the lens of conflict over fitness gains and competition for limited resources. The need for interaction between two sexes for reproduction can create a situation of sexual conflict between males and females, who have been selected to maximize their own fitness even if it generates a cost to the fitness of the other partner (Arnqvist and Rowe 2005; Moore and Pizzari 2005; Parker 2006; Chapman 2006). An example of such conflict is the evolution of traumatic copulatory appendages in male beetles, that damage the female reproductive tract but protect the male's sperm investment by making his mate less likely to mate with other males (Rönn et al. 2007). An additional consequence of considering reproduction as a social phenomenon is that reproductive phenotypes of an individual not only interact with those of the other sex, but also with those of group members (Moore and Pizzari 2005). The genotype of each individual also influences the social environment of other group members, including those of the opposite sex, creating new selection pressures; this phenomenon is termed indirect genetic effects (IGE;Wolf et al. 1998). As a result, the social environment can evolve more rapidly than the physical environment, leading to rapid changes in selection pressures. This perspective brings new predictions about the complexity of reproductive interactions: phenotypes that evolve under antagonistic interactions between males and females can enter a cycle of adaptation-counteradaptation (Moore and Pizzari 2005), and phenotypic changes in a group member create a new social environment for all other group members (Wolf et al. 1998). Sexual conflict and IGEs are predicted to lead to unusual levels of elaboration, plasticity and diversification in communication systems and their underlying sensing mechanisms (West-Eberhard 2014). Research on reproduction must thus expect complexity in the ecological, evolutionary and sensory mechanisms that have led to observed reproductive strategies and phenotypes.

Here we hope to illustrate the influence of ecological conditions and social context in modulating reproduction by reviewing current knowledge of the mechanisms that modulate the reproductive behavior of female *Drosophila melanogaster* fruit flies, in response to its environment. Most previous studies of reproductive behavior in *Drosophila* have been performed under reduced ecological complexity in the laboratory and have focused on males. However, this situation is rapidly changing with a recent increase in studies that consider the ecology of fruit flies when formulating hypotheses about environmental factors that affect behavior, and with a greater focus on females. Such experiments reveal that females are equipped with complex chemical senses that detect chemical substances emitted by their social and ecological milieus that change their behavior and physiology. Those substances, collectively called semiochemicals, include pheromones, which are chemical substance displayed by an individual that affect the behavior or physiology of others, as well as chemicals produced by food sources that act as cues of nutrient availability. Here, we review recent advances regarding the influence of semiochemicals on *Drosophila melanogaster* female reproduction. The genetics of this model organism, and the focus of many labs on its biology, has allowed dissection of how it senses chemical cues and how those affect behavior. We hope that this review will inform the development and testing of new hypotheses by neurogeneticists, behavioral geneticists, and chemical ecologists and will stimulate further integration between these two fields.

To provide the context for our review, we first outline *Drosophila melanogaster*'s life cycle in an ecological context. Although these flies are primarily associated with fruits, they are not, strictly-speaking, phytophagous insects. They feed upon the microbial community (bacteria and fungi) responsible for fruit decomposition, as well as upon the decomposed fruit itself. *Drosophila* thus live in an intensely smelly environment (Mansourian and Stensmyr 2015), full of chemical cues that modulate reproduction. Flies are attracted to ripe fruits by the volatile fermentation products produced by yeasts breeding on the fruit, and are sexually stimulated by the smell of yeast (Becher et al. 2010; Gorter et al. 2016; Grosjean et al. 2011; Palanca et al. 2013; Scheidler et al. 2015). Utilization of yeast smell to locate a source of this fungus and to mate in its vicinity is likely due to the strong dependency of *Drosophila* reproductive success on yeast. For example, *Drosophila* larvae will not develop in absence of key nutrients, such as sterols, that they normally obtain from yeast (Baumberger 1917; Carvalho et al. 2010). Female flies also require a rich diet to produce eggs (Bownes & Blair 1986) needing proteins, lipids, and sugars that can be supplied by yeast (Bownes, Scott & Shirras 1988; Carvalho-Santos and Ribeiro 2017). This strong dependency on yeast appears to drive much of *Drosophila* reproductive strategy and life-cycle: from egg-laying on fermenting substrates, to aggregation in great numbers on fermenting fruits where the social and sexual interactions that lead to mating and the start of a new cycle. This attraction to yeast also exposes *Drosophila* to competitors, such as microorganisms that compete with yeast for fruit resources, and parasitoids that prey on *Drosophila* larvae. Figure 1 outlines the steps of *Drosophila melanogaster* female reproduction and the web of ecological interactions and the chemicals that influence it, as will be discussed in this review.

1. FEMALE-DERIVED PHEROMONES THAT MEDIATE VIRGINS' ATTRACTIVENESS

Virgin *Drosophila melanogaster* females display over 50 different types of hydrocarbon and fatty acid molecules on their cuticle (Dweck et al. 2015b; Everaerts et al. 2010; Lin et al. 2016; Yew et al. 2009; Yew and Chung 2015). These molecules, collectively called cuticular hydrocarbons (CHs), act as cues of sex and species identity as well as of age, microbiome status, nutritional state, and social context. The surface of a virgin female is thus a readout of her genetic identity as well as of her experience and context (Billeter and Levine 2013; Everaerts et al. 2010; Farine et al. 2012). We begin this review by exploring the function of these chemicals as pheromones in the reproduction of virgin females.

1a. Intrinsic cues (age, sex, hormones) regulate the CH profile of a virgin female

Drosophila melanogaster adult females emerge from their pupal case unready for reproduction. Their ovaries do not contain mature eggs (Bodenstein 1947), their cuticle is not yet hardened, and the females are typically not sexually receptive (Manning 1967) (Figure 1a). Their CHs have not yet acquired an adult quality, suggesting that CHs act as cues of females' reproductive readiness. Immature male and female flies display long chain CHs with multiple double bonds and methyl branches; there is no sexual dimorphism in CH expression at this stage (Arienti et al. 2010; Pechine et al. 1988). Oenocytes, which are subcuticular abdominal cells that produce CHs (Figure 1a), are still developing during this early adult stage (Chiang et al. 2016; Makki et al. 2014; Wicker-Thomas et al. 2015). Females' development is regulated by hormones that coordinate maturation of different parts of the female reproductive system. This is shown by the fact that genetic disruption of the Juvenile Hormone (JH) pathway or ablation of the corpora allata, the glands that produce JH, delay the maturation of both adult female CH production and oogenesis (Bilen et al. 2013; Bodenstein 1947; Soller et al. 1999). The steroid hormone ecdysone might also be part of this maturation process, as genetic disruptions in the ecdysone pathway lead to aberrant oenocyte maturation and survival (Chiang et al. 2016) and ecdysone levels change with age, correlating with CH maturation (Arienti et al. 2010; Wicker and Jallon 1995). Bursicon, a peptide hormone known for its effect on hardening and tanning of the cuticle shortly after adult emergence and on wing eversion (Honegger et al. 2008; Peabody and White 2013), increases CH production, suggesting that it plays a role in regulating the time course of CH maturation and/or accumulation on the cuticle (Flaven-Pouchon et al. 2016). CH maturation might be important for female choice, as males can coerce females to mate within the first 30 minutes after eclosion (Markow 2000; Seeley and Dukas 2011); these young females' inability to fly and their poor locomotion make them a prime target for males (Figure 1a). In this situation, the males do not display courtship. They just jump onto the females and mate, engendering a cost to the female because she cannot choose her mate. Such a lack of choice is consistent with the observation that these females will remate at higher frequencies when they become mature (Seeley and Dukas 2011). Maturation of the CH profile correlates with, and presumably induces, increased male courtship (Bilen et al. 2013). This increase in courtship by males can presumably give females more opportunities to evaluate the males and exert mate choice. That female CHs increase male courtship and delay mating is consistent with the observation that mature adult females lacking CH are

more attractive to males than are females with a fully developed CH profile (Billeter et al. 2009).

Females become reproductively mature two days post-eclosion. At this time their ovaries are mature, (Bodenstein 1947) and the females have higher mating receptivity (Manning 1967). These females also have mature oenocytes that produce ~50 different CHs, most of these shared with males, except for long chain dienes including (7*Z*,11*Z*)-Heptacosadiene (7,11-HD) (Everaerts et al. 2010;Figure 1b; Figure 2). Female-specific production of dienes makes these molecules attractive candidate sex pheromones, but this role is not yet completely established. Mature females lacking CHs, including 7,11-HD, due to genetic ablation of the oenocytes, are preferred over control females as mating partners by males from some strains but not by others (Billeter et al. 2009; Pischedda et al. 2014). This means that for some strains, CHs can lower attractiveness, whereas in others CHs increase it, illustrating the importance of CHs as pheromones that regulate intra-specific courtship. The recent discovery of fatty acid-derived pheromones, such as methyl laurate (Figure 2), that are found on the cuticle of flies but are not produced by oenocytes, might shed light on how CH tune female attractiveness (Dweck et al. 2015b; Lin et al. 2016). These fatty acid-derived pheromones are found on both males and females of most *Drosophila* species and seem to function as general sex pheromones that stimulate male courtship. Methyl laurate, as well as a few other fatty acid-derived pheromones, is detected by olfactory receptor Or47b; males unable to sense this compound reduce courtship towards females (Dweck et al. 2015b; Lin et al. 2016). These fatty acid-derivatives likely act as general attractive sex pheromones in many *Drosophila* species, whereas sex- and species-specific CH made by the oenocytes likely mitigate the effects of these attractive pheromones both intra- and inter-specifically (Billeter et al. 2009; Savarit et al. 1999; Figure 1b). Indeed, the most striking and consistent phenotype of *D. melanogaster* females lacking CHs is that they are intensely courted by males of other species (Billeter et al. 2009; Savarit et al. 1999), an effect that can be countered by applying small amounts of synthetic 7,11-HD to CH-less females (Billeter et al. 2009). This shows that in addition to its pheromonal effects within *D. melanogaster*, female-specific 7,11-HD deters courtship by heterospecific males (Figure 1b).

A close look at the CH displayed by different *Drosophila* species reveals that males' CH are generally qualitatively invariant, but that there are large inter-species differences in CH displayed by females. For example, 7,11-HD is a female-characteristic CH only in D*. melanogaster*. Females of other species, like *D. erecta,* make the long chain diene (9*Z*, 23*Z*)-Tritriacontadiene, and females of species like *D. simulans* make monoenes, such as (*Z*)-7-Tricosene (7-T)(Jallon and David 1987; Figure 2). The prominent female CH from each species appears to be the pheromone that blocks inter-species mating; for instance 7-T appears to be the pheromone that normally blocks *D. melanogaster* males from courting *D. simulans* females, as *D. melanogaster* males mutant for the putative 7-T receptors Gr32a court *D. simulans* females (Fan et al. 2013), and *D. melanogaster* females lacking 7,11-HD are courted by males from other *Drosophila* species (Billeter et al. 2009). Differences in female CH expression arise from regulatory changes in the expression of CH-metabolic enzymes. 7,11-HD is synthesized in *D. melanogaster* female oenocytes through the action of a series of enzymes including *desaturase1 (desat1)* (Marcillac et al. 2005), *desaturaseF* (Chertemps et al. 2006), and *elongaseF* (Chertemps et al. 2007). The enzymatic pathway that

controls 7,11-HD synthesis is expressed differently between species. For example, *D. melanogaster desaturaseF,* a gene necessary for 7,11-HD synthesis, is expressed specifically in females. However across several *Drosophila* species, the promoter of the *desaturaseF* gene shows repeated loss and gain of the sex-specific Doublesex-binding element, resulting in the gene's expression in both males and females of some species (Shirangi et al. 2009). There is also variation in the amount of two heptacosadiene (HD) isomers between *D. melanogaster* populations: 7,11-HD is predominant in most laboratory strains, whereas 5,9-HD levels are relatively high in Caribbean and sub-Saharan African strains (Ferveur et al. 1996). The 7,11-HD:5,9-HD ratio is controlled by two closely related genes, *desat1* and *desat2*, (Coyne et al. 1994; Dallerac et al. 2000). A deletion in the promoter of *desat2* has been correlated with high 7,11-HD and low 5,9-HD, whereby a more-active *desat2* promoter results in high levels of 5,9-HD (Takahashi et al. 2001). The functional consequence of this difference is unclear. Females with 7,11-HD generally mate faster than do females with 5,9-HD, perhaps because the former elicits more courtship by males (Ferveur et al. 1996). Taken together, these data indicate that *Drosophila* female CHs function to block inter-species mating, by blocking courtship from heterospecifics, as well as having a role in promoting intraspecific mating (Figure 1b).

The high molecular weights of female dienes such as 7,11-HD (Figure 2), make them unlikely to be volatile pheromones (Antony and Jallon 1982). Rather, they remain on the female's cuticle and are detected by males through contact taste receptors. The involvement of the male's taste system in detecting female sex-specific CHs has been clearly documented. Males sense female CH via gustatory neurons, in the males' first pair of legs, that express the ion channel *pickpocket 23 (ppk23)* (Lu et al. 2012; Thistle et al. 2012). Although *ppk23* is necessary for sensing 7,11-HD, it is unclear whether this ion channel is the receptor for this sex pheromone. It is a predicted sodium ion transporter, so it may act as an effector or amplifier of signal transduction downstream of chemoreceptors in taste neurons dedicated to pheromone sensing (Thistle et al. 2012). Although taste-sensing is clearly critical for detecting 7,11-HD, earlier work suggested the existence of volatile female compounds (Tompkins et al. 1980; Tompkins and Hall 1981), whose chemical identity remained undefined until recently. In 2017 Lebreton *et al*. discovered that 7,11-HD is oxidized quickly when in contact with air, generating a volatile degradation product (*Z*)-4-undecenal (Figure 2). This latter molecule is sensed by odorant receptor Or69a and can elicit upwind flight from both male and female *D. melanogaster*, but not from *D. simulans*. Thus, female *D. melanogaster* make a species-specific volatile pheromone that attracts conspecific males (Lebreton et al. 2017). It remains to be seen whether this pheromone also elicits courtship, or whether that is the role of the intact 7,11-HD. Resolution of this question will shed light on the relative role of olfactory versus gustatory detection of sex pheromones.

1b. Extrinsic cues (diet, daily cues, environment) also regulate the CH profile of a virgin female

D. melanogaster males mate preferentially with more fecund females (Arbuthnott et al. 2017), suggesting that females display cues of their fecundity. Although visual cues such as size are probably important in mate-assessment (Laturney and Billeter 2014), it appears that pheromones also play a role (Arbuthnott et al. 2017). Indeed, the pheromones displayed by a

female are responsive to external cues, providing information about the female's ecological context and thus her potential suitability as a mate. Three examples of this relate to her nutritional status, her circadian status and social environment, and the microbial environment in which the female is situated.

A central nutrient-sensing pathway, insulin signaling, affects CH production and female attractiveness (Kuo et al. 2012). Insulin signaling also regulates absorption of nutrients by the fat body. This tissue produces yolk proteins that will be taken up by the developing oocyte; yolk protein production is thus a major limiting step in oocyte maturation and hence fecundity (Fedina et al. 2017). Interestingly, under conditions of low insulin, where resources in the fat body may be released to fuel egg production, the females' oenocytes reduce production of the CHs 7-T and (*Z*)-5-Tricosene (5-T)(Fedina et al. 2017). This is intriguing because these two CHs, which are normally produced at higher levels in males than in females, had previously been associated with reducing male-male courtship, suggesting that they may play a role in decreasing the attractiveness of any fly to a romantically-inclined male (Billeter et al. 2009; Ferveur and Sureau 1996; Lacaille et al. 2007; Wang et al. 2011). Thus, a reduction in the quantity of these courtship-inhibitory pheromones displayed on the female cuticle should lead to increased attractiveness of that female (Figure 1b). This correlation between the effect of insulin on oocyte production and on the level of inhibitory CH suggests that males could use a female's CH profile as a proxy to sense her nutrient levels and, indirectly, her fecundity. Information about the nutritional state of a female, and hence her ability to produce eggs, may thus be cued by the same pheromone that increases her attractiveness.

CH production in mature adult females is also influenced by the circadian clock system that synchronizes the fly's behavior and physiology to day/night length and to the activity of other flies (Levine 2002; Hall 2003). Flies' CH profile changes throughout the day under the influence of central brain clock neurons that entrain to light conditions (Krupp et al. 2008). These neurons send information to the oenocytes through the neuropeptide Pigment Dispersing Factor to affect expression of genes that regulate CH synthesis (Krupp et al. 2008; Krupp et al. 2013). It seems likely that flies use circadian fluctuations in pheromone profiles to detect information about the phase of activity of other flies, since sensing the air from flies exposed to normal light:dark conditions can entrain the circadian clock of flies living in complete darkness (Levine 2002). The phase information provided by the CH profile could help flies synchronize their activity with one another. Strikingly, the circadian system also regulates CH production in the context of another external cue, the genotype of group members. The genotypic composition of males in a group affects the expression of CH synthetic enzymes in the oenocytes of other males, and thus CH production. This too is regulated by the circadian-gene system, which presumably detects environmental changes in social context in addition to its known function in adapting to changes in abiotic factors, such as photoperiod (Bloch et al. 2013; Kent et al. 2008; Krupp et al. 2008; Krupp et al. 2013). That pheromones displayed by one individual are affected by the genotype of others is an illustration of IGEs (Kent et al. 2008). These changes in male pheromone profiles might help females detect genetic diversity in nearby males. Indeed females mate more frequently when surrounded by genetically diverse males, whose CH production is modulated by one another's presence (Billeter et al. 2012; Kent et al. 2008; Krupp et al.

2008;). The genetic composition of a group thus influences male pheromones, which in turn can influence a female's mating decision. This will ultimately affect the genetic composition of the next generation, and thus the evolutionary process.

The pervasiveness of the ecological context in determining a fly's pheromonal profile is probably even broader. Bacteria living on the food consumed by *Drosophila* can become part of the fly's microbiome (Sharon et al. 2010), and through as yet unknown mechanisms, can affect their CH profiles. Some studies have proposed that this can lead to assortative mating between males and females raised on the same diet and infected by similar bacteria (Sharon et al. 2010). However, a new study presents data that challenge this interpretation (Leftwich et al. 2017). Pathogenic bacteria can also increase the production of *Drosophila* aggregation pheromones. These pheromones attract flies to the area, and flies that were uninfected can then become infected (Keesey et al. 2017).

Thus, the chemical profile of a virgin female fly reflects not only her sex, maturity, and species, but also her nutritional status, social context and experience (including mating status, see later section). We hope that future work will highlight more of the mechanism through which a fly's biography can be displayed on her cuticle and how other individuals can use this information to instruct their social interactions with that individual.

2. PHEROMONES TRANSFERRED BY MALES TO FEMALES DURING MATING AND THEIR FUNCTIONAL CONSEQUENCES

2a. Cuticular Hydrocarbons

Male *D. melanogaster* also produce and display cuticular hydrocarbons. In particular, their oenocytes make 7-T (which females also display on their cuticles, including in response to nutrient cues (see above) but in much smaller quantities). When flies mate, the 7-T from the male rubs off onto the female, where it remains for at least two days and probably longer (Everaerts et al. 2010; Scott 1986; Yew et al. 2009). This additional 7-T on the female decreases her attractiveness immediately after mating to other males, which detect the molecule with gustatory receptor Gr32a on their forelegs (Laturney and Billeter 2016; Miyamoto and Amrein 2008; Wang et al. 2011) as well as Gr66a (Lacaille et al. 2007). Several other male CH are transferred to females during mating, but they have not yet been shown to have an effect on the female's attractiveness (Everaerts et al. 2010; Laturney and Billeter 2016).

2b. Molecules in seminal fluid

In addition to transferring CH to females via cuticular contact, male *D. melanogaster* also make a fatty acid pheromone, cis Vaccenyl Acetate (cVA) that they transfer to females during mating by a different means (Butterworth 1969). cVA is made by a male reproductive tissue, the ejaculatory bulb (Brieger and Butterworth 1970; Guiraudie-Capraz et al. 2007), and is transferred to female in the seminal fluid (Figure 2). cVA, sensed by males' olfactory receptor Or67d, reduces male courtship (Ejima et al. 2007; Ha and Smith 2006; Kurtovic et al. 2007; Ronderos and Smith 2010; van der Goes van Naters and Carlson 2007). cVA might be also sensed by gustatory receptors in males' legs (Thistle et al. 2012). Finally, the

pheromone (3R,11Z,19Z)-3-acetoxy-11,19-octacosadien-1-ol (CH503), is also transferred to females during mating; like cVA, it is made in the ejaculatory bulb and reduces male courtship (Ng et al. 2015; Yew et al. 2009); males sense this pheromone via gustatory receptor Gr68a (Shankar et al. 2015; Yew et al. 2009;). Thus, females receive several hydrocarbon and fatty acid-derivative pheromones from their mates via cuticular transfer and via seminal fluid (Figure 1c), and these pheromones affect the females' attractiveness (Figure 1d). We will see in section 3b that 7-T and cVA work synergistically, in a blend, to decrease the female's attractiveness to other males.

Most pheromones are relatively small molecules, including volatile molecules that are released into the environment or CHs that are displayed on the surface of an animal (e.g. see above; Yew and Chung 2015). However, a class of much larger and non-volatile molecules that are present in the semen of *D. melanogaster* males falls under the definition of pheromone as well. These molecules are transferred to the reproductive tract of females during mating (Figure 1C) and influence the female's behavior and physiology (Avila et al. 2011; Hopkins et al. 2017; Perry et al. 2013; Figure 1d). *D. melanogaster* males produce hundreds of Seminal Fluid Proteins and peptides (SFPs) whose effects transition females from virgin to reproductive states. Behavioral changes induced by these SFPs in females include diminished sexual receptivity (Avila et al. 2011; Chapman et al. 2003; Chen et al. 1988; Liu and Kubli 2003), increased protein feeding (Carvalho et al. 2006; Ribeiro and Dickson 2010), decreased sleep (Dove et al. 2017; Garbe et al. 2016; Isaac et al. 2010), and increased aggression (Bath et al. 2017). Physiological changes include increased egg production and ovulation (Chapman et al. 2003; Heifetz et al. 2000; Liu and Kubli 2003; Rubinstein and Wolfner 2013), changes in gut size and in the rate of food transit through the gut (Apger-McGlaughon and Wolfner 2013; Cognigni et al. 2011; Hudry et al. 2016; Reiff et al. 2015), changes in vesicle release and in neuromodulator levels in the reproductive tract (Heifetz and Wolfner 2004; Heifetz et al. 2014), and conformational changes of internal organs such as the oviducts (Rubinstein and Wolfner 2013) and uterus (Adams and Wolfner 2007; Avila and Wolfner 2009), likely due to muscle contraction/relaxations. During mating, several SFPs enter the circulation of the female by crossing the wall of the very distal part of her reproductive tract (Lung and Wolfner 1999). Recent studies have shown that the male's intromittent organ punctures the intima of the female reproductive tract, potentially providing a direct route for SFPs to enter the circulation (Kamimura 2007; Mattei et al. 2015; Mattei et al. 2017). From the circulation SFPs can reach all tissues of the female, including her neuroendocrine systems. Although the following paragraphs focus on *D. melanogaster* SFPs, seminal molecules with "pheromonal" effects need not be peptides or proteins. For example, in certain mosquitoes, the sesquiterpenoid juvenile hormone (JH; Aedes; Borovsky et al. 1994; Feinsod and Spielman 1980; Klowden and Chambers 1991; Shapiro et al. 1986) or the steroid hormone ecdysone (Anopheles) induce post-mating changes in behavior or physiology (Gabrieli et al. 2014).

Perhaps the best known of the *D. melanogaster* seminal pheromones is the "Sex Peptide" (SP), a 36-amino acid long (Figure 2) peptide that is produced in the male's reproductive accessory glands, and transferred to the female during mating. Different regions of SP exert different effects on the female. SP's N-terminal region increases JH production by the female's corpus allatum, as shown both in tissues incubated with SP (where JH BIII

production is increased by SP (Moshitzky et al. 1996)), and *in vivo* (Bontonou et al. 2015; Soller et al. 1999). JH, in turn, is needed for the increased oogenesis that occurs after mating. SP's C-terminal region stimulate a decrease in female sexual receptivity starting a few hours after mating (Chapman et al. 2003; Liu and Kubli 2003; Peng et al. 2005). The C-terminal region of SP also stimulates oogenesis (Peng et al. 2005), excretory characteristics (Apger-McGlaughon and Wolfner 2013), and sperm release from storage (Avila et al. 2010). It is possible that this region is also sufficient to cause post-mating changes in siesta sleep (Dove et al. 2017; Garbe et al. 2016; Isaac et al. 2010), feeding (Carvalho et al. 2006; Ribeiro and Dickson 2010), and aggression (Bath et al. 2017) but these have not been tested directly. The C-terminal portion of SP acts through a G-protein coupled receptor, the Sex Peptide Receptor (SPR), which is found in the nervous system and elsewhere in the female including in her sperm storage organs (Yapici et al. 2008). Evidence has been presented for the existence of a second SP receptor whose molecular identity is as yet unknown (Haussmann et al. 2013). Genetic studies have defined neurons through which SP acts, via SPR, to affect female sexual receptivity behavior and egg-production; it is possible that it also acts through these neurons to induce the other behaviors (Feng et al. 2014; Hasemeyer et al. 2009; Hussain et al. 2016; Ottiger et al. 2000; Rezával et al. 2012; Rezával et al. 2014; Walker et al. 2015; Yang et al. 2009). SP's effects on sperm release from storage requires SPR in both the nervous system and the sperm storage organs (Avila et al. 2010; Avila et al. 2015b).

As with all other seminal proteins tested (Ravi Ram et al. 2005; Monsma et al. 1990), SP remains in the female's circulation for less than a day; it is degraded by proteases it encounters there (Pilpel et al. 2008), however, SP's effects on females' s receptivity and egg-laying, for instance, persist for much longer: 10–14 days. Peng et al. showed that the effects of SP are maintained because the peptide binds to sperm (via its N-terminal region), apparently protecting it from degradation once SP bound sperm are in storage (Peng et al. 2005). A cascade of seminal proteins is needed to bind the SP to sperm (Findlay et al. 2014; Ram and Wolfner 2009). SP's C-terminus is cleaved from sperm by a trypsin-like activity (Peng et al. 2005), releasing it to bind to its receptor SPR and to induce the behavioral and some long-term physiological changes.

Other seminal proteins also influence the female's physiology, thus also qualifying them as pheromones. For example, the SFP prohormone ovulin enters the female and stimulates octopaminergic signaling on her reproductive tract musculature (Rubinstein and Wolfner 2013), relaxing her oviduct and stimulating the ovulation of oocytes. Another seminal protein, Acp36DE, affects the conformation of the female's uterus (Avila and Wolfner 2009), presumably by regulating contraction of the muscles encircling it; this is suggested to facilitate the movement of sperm towards storage sites.

In summary, males transfer a complex mixture of pheromones to females during mating. These molecules are made in the oenocytes and in the reproductive tract, and can belong to completely different chemical classes (Figure 2). Despite these differences, these pheromones have in common two remarkable features: they are not transferred via the air but via contact or via semen, and they act on females to induce a transition from a virgin to a reproducing state. They effect this state change by both acting on the physiology and

behavior of the female and by indicating to others that the female has mated by changing her pheromonal profile.

3. EVOLUTIONARY CONSIDERATIONS REGARDING PHEROMONES

A female's sex pheromones play a critical role in broadcasting her age, species, mating-appropriateness and condition, and male pheromones that are transferred to her modulate her behavior, physiology, and attractiveness. It is interesting to consider these roles in light of the species-specificity required for successful reproduction, as well as the dynamic of conflicts in reproductive strategies of males and females. Several important areas reflect, or are influenced by, the roles of pheromones in this evolutionary context.

3a. Pheromones display high species-specificity

Female CH pheromones are often highly species-specific (Jallon and David 1987), attracting only conspecific males to the female for mating. Likewise, the primary amino acid sequences of an unusually high number of seminal proteins have evolved rapidly (Swanson 2004; Haerty et al. 2007), giving rise to species-specific sequences. Seminal proteins that show signs of positive selection at the amino acid level, or other evidence of positive selection, include ovulin (Aguadé et al. 1992), Acp36DE (Begun et al. 2000), and SP (Cirera and Aguadé 1997). Ovulin orthologs are difficult to find outside the *melanogaster* group (Mueller et al. 2005), and it is not yet known if those potential orthologs – some different enough not to cross-react with anti-*melanogaster*-ovulin - are functional. SP orthologs are found throughout *Drosophila*, though distant species' are very different in sequence and are not functional in *D. melanogaster* (Tsuda et al. 2015; Tsuda and Aigaki 2016). Thus, both ovulin and SP appear to be novel genes, that have evolved rapidly. Interestingly the SPR is highly conserved and found in many insect orders such as mosquitoes, Lepidoptera and beetles (Yapici et al. 2008). The ancestral ligands for this receptor are believed to be the well conserved myoinhibitory peptides, whose functions include regulating muscle contraction and fluid balance (Kim et al. 2010; Yamanaka et al. 2010). It appears that SP arose and coopted the receptor for myoinhibitory peptides, and then was selected for in certain *Drosophila* lineages as it conferred advantages to the male in preventing his mate from remating and in inducing his mate to undertake physiological and behavioral changes that can increase her progeny production. While it is likely that the rapid evolution of seminal proteins arose due to conflicts between the reproductive interests of males and females (e.g. Sirot et al. 2014), one could imagine potential contributions from a Muller-Dobzhansky system, in which protein partners that co-evolve within a lineage become incompatible with their counterparts in a different lineage (see Maheshwari and Barbash 2011 for review). Moreover, the rapid evolution of seminal proteins may also have been favored as part of a fail-safe mechanism: if pre-mating mechanisms like pheromones (and other cues) failed to maintain species isolation, the species-specificity of seminal proteins could help keep an interspecies mating unsuccessful.

3b. Pheromones often act in blends

While precise chemical nature of a pheromone can confine its effect to a single targeted species, further specificity can be gained if a successful signal involves a combination of

molecules, in particular concentrations, e.g. a blend. This idea is well documented in the sex pheromones of *Lepidoptera* (Renou 2014), but is also is important in CH of *D. melanogaster* and, likely, in SFP blends in this species.

For example, as noted above, *D. melanogaster* males rub the CH 7-T (Figure 1c; Figure 2) onto females during mating and also transfer the volatile pheromone cVA to females in semen. These two pheromones act as a blend to reduce female attractiveness (thus acting as fast-acting mate-guarding pheromones that help the male protect his sperm investment; see below). The 7-T/cVA blend of mate guarding pheromones makes females smell partially like males. Virgin females do not produce cVA and only exhibit small amounts of 7-T, while males produce these two molecules in high quantities (Everaerts et al. 2010). That 7-T and cVA act as a blend to reduce male courtship is evident in that males devoid of CH, including 7-T, but who still produce cVA, are attractive to other males (Billeter et al. 2009). cVA is thus not sufficient to reduce male-male courtship. Rather, it requires 7-T as a partner for its mate-guarding effect, as perfuming males lacking CH with 7-T blocked courtship from other males (Billeter et al. 2009; Wang et al. 2011). In keeping with the idea that males "masculinize" the pheromone profiles of females during mating, perfuming virgin females with only 7-T or cVA at their natural dose does not decrease in females' attractiveness. It is only when both pheromones are present that they, together, cause decreased female attractiveness (Laturney and Billeter 2016)- the blend is uniquely effective.

By marking their mates with cVA and 7-T, males make these females smell like males (Scott 1986). Such a trick, in conjunction with mated females having lower sexual receptivity than virgins (Chen et al. 1988; Manning 1967), explains why males have not evolved to ignore this mate-guarding pheromone. Even though ignoring that pheromonal combination could lead them to sire most of the offspring from previously-mated females, ignoring it would also lead them to court males, a strategy with low fitness benefits. Interestingly, in addition to donating anti-aphrodisiac pheromones (7-T, cVA) to mated females, males also reduce females' expression of attractive sex pheromones such as 7,11-HD. Specifically, the seminal pheromone SP down-regulates females' hydrocarbon (CH) production (Bontonou et al. 2015). Thus, males lower female attractiveness by marking them with male pheromones and by altering females' production of sex pheromones. That male hydrocarbon pheromones make females unattractive immediately after mating, may buy time for SP to act via the receptors and neural pathways noted above to decrease female receptivity.

Blends may also be important in the optimal action of SFP pheromones. Male *D. melanogaster* appear to be capable of adjusting the amounts, and relative levels, of SFPs in response to social cues (related to perceived sperm competition risk. In the presence of rivals, males transfer more seminal proteins (ovulin and SP) and sperm (Garbaczewska et al. 2012; Perry et al. 2013; Sirot et al. 2011; Wigby et al. 2009; Wigby et al. 2016), thus transferring ejaculates of different composition in response to sperm competition risk (Parker and Pizzari 2010). In addition, when males are mated with previously mated females, they transfer more SP relative to ovulin than when they mate with previously unmated females (Sirot et al. 2011). Thus, males can adjust the blend of seminal proteins transferred when faced with a mated female, increasing the relative amount of a paternity-protecting molecule (SP) relative to the amount of a molecule (ovulin) that had already acted

and on whose prior activity they can piggy-back. The idea of a different blend for different circumstances recalls the different pheromone blends used by different species for mate-attraction.

3c. Pheromones may act as signals or as switches (on/off or rheostats)

Pheromones change the characteristics, and in some cases the behavior and physiology, of the recipient. In some cases, they do so directly, acting as signals at the top of a signal transduction pathway –e.g. as ligands for odorant (or taste) receptors whose activated state is then transduced to the sensory system (Gomez-Diaz and Benton 2013). For example, the hydrocarbon and semen pheromones discussed above that are transferred by male *D. melanogaster* change a mated female's smell and taste to more male-like, resulting in her decreased attractiveness to other males, without any direct action by the female – the compounds simply act as ligands for the male's receptors.

In other cases, pheromones switch the recipient to a new state by hijacking pre-existing hormonal or sensory pathways in the recipient. This is particularly the case for the SFP pheromones. Here too however, as with the ligands just mentioned, the SFPs appear to act at the tops of pathways – in this case as switches that regulate the activity of a pre-existing pathway in the female. Sometimes SFPs can act as traditional on/off switches. For example, ovipositor extrusion is only seen in mated females; this behavior is turned "on" (from an "off" state) by SP (Chen et al. 1988). In other cases, SFPs act more like rheostat-switches, turning up (or down) pre-existing pathways and processes in mated females. Examples are that ovulin increases octopaminergic signaling (and thus ovulation) in mated females (Rubinstein & Wolfner, 2013), and that SP increases oogenesis and decreases receptivity by mated females (Chapman et al. 2003; Chen et al. 1988; Liu & Kubli, 2003). In all of these cases, the process seen post-mating (ovulation, oogenesis, occasional low receptivity) occurs to some low level in virgin females, but the SFP acts to increase its level, often greatly.

3d. Sexual conflict is important to consider when evaluating pheromones' mechanisms

Thus far, we have discussed mechanisms of pheromone perception as important in initial proximate interactions between females and males, and have noted that male CH pheromones and SFPs also can affect the likelihood of a mated female mating with another male. This latter effect arises because polyandry is costly to males in species like *D. melanogaster*, due to last male sperm precedence (Manier et al. 2010; Parker and Pizzari 2010; Schnakenberg et al. 2012). This phenomenon, in which the last male to mate sires the majority (often 80–90%) of offspring, means that a male's reproductive success will be disadvantaged if his mate mates again (Parker and Pizzari 2010). Sexual conflict theory predicts that *Drosophila* males should evolve mechanisms that either reduce their partners' ability to re-mate ("mate guarding") and/or increase the chances of the female using his sperm over that of another male. As noted above, hydrocarbon pheromones perform mate-guarding functions, as does SP's inhibition of female receptivity. Other SFPs affect sperm competition outcomes (Chapman et al. 2000; Clark et al. 1995; Fiumera et al. 2005; Harshman and Prout 1994; Reinhart et al. 2014; Zhang et al. 2013), consistent with the effects on sperm use but are beyond the scope of this review. However, polyandry can be beneficial for females (Arnqvist and Nilsson 2000; Jennions and Petrie 2000;), for example

by allowing them to acquire sperm from different males, with advantages in producing the most-fit progeny. This sets up a tension between the strategies/needs of females and males, leading to sexual conflict and an arms race. Simply put, males transfer pheromones that act to their advantage, and females acquire resistance to those pheromones, to their advantage. Males can then evolve new pheromones or other mechanisms to overcome female resistance.

Such conflicts have been suggested to have driven some aspects of the CH pheromone phenomenon in *D. melanogaster* (Laturney and Billeter 2016), and at least some of the rapid sequence evolution of critical *D. melanogaster* seminal proteins (Sirot et al. 2015). Focusing on the former, the transfer of 7-T and cVA by males reduces the female's chance of remating, which can reduce her potential to accrue fitness benefits. In such a situation, females that remove or deactivate those pheromones, thus restoring her attractiveness and/or remating propensity, will accrue fitness advantages. Recently it was shown that females exhibit a behavior that can mitigate the effect of mate guarding hydrocarbon pheromones and SFPs that affect her remating receptivity. A few hours after mating, females eject the surplus male ejaculate located in their uterus (bursa), including its cVA and possibly a large fraction of SFPs (Figure 1e; Dumenil et al. 2016; Laturney and Billeter 2016; Lee et al. 2015; Manier et al. 2010). This ejection removes ~90% of cVA, altering the blend with 7-T that had led to optimal mate–guarding, and thus restoring some female attractiveness (Laturney and Billeter 2016). Therefore, male manipulation of female attractiveness can be disrupted via ejaculate ejection, a typical post-mating behavior.

The cVA that males provide to females is ejected with the mating plug that formed at the entrance of her reproductive tract. This gelatinous plug is a coagulation of proteins from male reproductive glands (such as accessory glands and ejaculatory bulb), and female proteins (at least in mosquitoes (Rogers et al. 2009), and fills the bursa of the mated female. It is thought to help retain ejaculate, including pheromones like cVA in the female's bursa and may also assist sperm movement into storage. Prevention of *D. melanogaster* mating plug coagulation by knockdown of a critical mating plug protein from the male's ejaculatory duct (pEBme)(Avila et al. 2015a) results in premature loss of ejaculate from the female, minimizing sperm storage and affecting post-mating responses that depended on stored sperm. Moreover, at least one mating plug protein affects female remating behavior before mating plug ejection (Bretman et al. 2010), in addition to the effects of cVA. Indeed nearly half of the recently ejected females re-mated during a 30-minute observation period, whereas all the non-ejected females abstained (Figure 1e,f; Laturney and Billeter 2016). This finding suggests a close temporal relationship between ejection and remating: females that are faster to eject may also be faster to re-mate. The timing of ejaculate ejection is plastic. It is also socially modulated: females that were held in groups ejected the ejaculate 1 hour earlier than females that had mated in single-pairs and were then isolated after copulation (Laturney and Billeter 2016). As females also mate faster and more often when in social contexts that contain more flies and with more genetic diversity (Billeter et al. 2012; Gorter et al. 2016; Krupp et al. 2008; Laturney and Billeter 2016), females may be able to modulate timing of ejection to influence attractiveness in order to maximize reproduction. In contexts that are favorable for their reproduction, such as when genetically diverse males are present, females may shorten their ejection latency in order to attract potential mates and increase genetic diversity of offspring. However, if remating is not likely or beneficial, such as when

the female is isolated or is with inbred males, females may lengthen their ejection latency in order to reduce unwanted sexual harassment or to ensure full usage of their already obtained ejaculate.

The continued evolutionary interplay between female and male reproductive strategies and molecules may explain why pheromones such as SFPs often act as switches (on/off or rheostats, as discussed above). Physiology and behavior are regulated by complex molecular pathways that often involve the action of pleiotropic molecules and conserved machinery (for example, ovulation in both *Drosophila* and locust require octopamine signaling in females; Monastirioti et al. 1996; Monastirioti 2003; Lange 2009). In this context, it may be simpler to evolve a new switch to turn on/off (or up/down) a pathway (e.g. ovulin in *D. melanogaster*) if the old switch has become ineffective due to counter-evolution, rather than to evolve a new or modified internal component of the intricate and pleiotropic pathway.

4. INFLUENCE OF THE ECOLOGICAL CONTEXT ON FEMALE REPRODUCTIVE BEHAVIORS

So far in this review, we have focused on chemicals that guide sexual interactions between flies. Ecological conditions, such as food availability and the presence of harmful organisms, can have dramatic effects on female reproductive behaviors. Interestingly those conditions mainly affect mated and not virgin females. They are sensed through chemical cues produced by the food sources, predators and pathogens. The female's reactions to those semiochemicals function to ensure offspring survival and might thus be considered a primitive form of maternal care.

4a. Role of food in female post-copulatory sexual receptivity

Food availability is paramount to female reproduction because it provides the energy for both the production of eggs and the survival of offspring during development. As we have seen above, male seminal pheromones transferred to females during mating stimulate egg production, which puts high nutritional demands on females. These nutritional needs are accompanied by a change in female physiology, including a change in intestinal morphology and function (Apger-McGlaughon and Wolfner 2013; Cognigni et al. 2011; Lemaitre and Miguel-Aliaga 2013; Reiff et al. 2015) as well as a change in the female's diet. Mating shifts the dietary preference of female flies from sugar-rich food to yeast, which is a prime source of proteins (Carvalho et al. 2006; Ribeiro and Dickson 2010; Vargas et al. 2010). This shift in dietary preference towards yeast is adaptive, as the protein content of yeast fuels egg-production and yeast itself is necessary as a food source for offspring development and survival (Baumberger 1917; Terashima and Bownes 2004). Interestingly, these changes in food preference are not controlled directly by the demands of egg-production, as mated females lacking oocytes still consume more yeast (Barnes et al. 2007; Ribeiro and Dickson 2010). Instead this dietary change is triggered by SP. This male pheromone can shift a female's nutritional preferences towards a diet that benefits offspring production and survival (Barnes et al. 2008; Carvalho et al. 2006; Ribeiro and Dickson 2010; Vargas et al. 2010).

Yeasts produce several volatile fermentation products (Becher et al. 2012) that act as chemical cues that stimulate sexual activity, coupling a cue about the presence of an important nutritional resource for reproduction with sexual arousal. This simple coupling is seen for *D. melanogaster* males, whose courtship intensity increases in the presence of odors from food (Grosjean et al. 2011). Interestingly, the food odor-cue that triggers increased male courtship is sensed by olfactory neurons whose second order neuronal projections converge with those of sex pheromone sensing neurons, making the smell of certain foods akin to pheromones in both function and sensory mechanism (Grosjean et al. 2011), in contrast to the situation with other (general) odors. The mating propensity of females also changes when there is yeast in their diet (Fricke et al. 2010; Harshman et al. 1988): presence of yeast increases the sexual receptivity of mated (but not virgin) females (Figure 1f; Gorter et al. 2016). However the coupling of food (yeast) odors to sex is not as simple for *D. melanogaster* females as it is for males: the smell of yeast is not sufficient to increase mated females' likelihood of remating (Gorter et al. 2016). Rather, the combination of yeast odors, in particular acetic acid (Figure 2), plus the presence of the yeasts' amino acids is required to trigger an increase in females' mating receptivity. The olfactory sensing of yeast relevant to sexual behavior is mediated by the ionotropic olfactory receptor family in both males and females, in particular Ir84a (Grosjean et al. 2011) in males and Ir75a in females (Gorter et al. 2016), but the identity of the receptor for yeast amino acids remains unclear. Mated females must integrate two signals, one received through olfactory means, the other probably through gustatory, to increase sexual receptivity in the presence of yeast. This integration allows mated females to simultaneously measure the consumed yeast nutritional resources required for egg production and the presence of environmental yeast that will be required for offspring growth. Such integration insures against the inability of the peripheral nervous system to discriminate substances that smell or taste like yeast but cannot be metabolized to fuel egg production. Finally, why does yeast affect the sexual receptivity of mated but not virgin females? Multiple mating is costly to females (Chapman et al. 1995; Fowler and Partridge 1989; Wigby and Chapman 2005), so mated females would maximize fitness by only mating to acquire more sperm or ovulation-boosting male seminal peptides when there are enough yeast resources for sustained egg production and for offspring development. In contrast, it is sufficiently important that a virgin female acquire sperm that she would not benefit from restricting her mating only to the most optimal, yeast-laden, environments.

4b. Role of chemical cues in egg-laying site choice

Female flies also rely on their sense of smell and taste to identify sites on which to lay their eggs. Selection of an egg-laying site occurs in two phases. First, females are attracted to a prospective oviposition site. Second, once there, females utilize different, more local, cues for the final decision to deposit eggs.

4b1. Chemical cues to find an appropriate egg-laying site—The first step in oviposition is locating an appropriate site (Figure 1g). Mated *D. melanogaster* females are attracted to decaying fruits (Becher et al. 2012; Reed 1938). Yet their "fruit fly" name is somewhat misleading, because they are actually attracted by the combination of fruit and the yeasts that grow on the fruits. Among volatile cues that attract flies are yeast fermentation products, and yeast alone is sufficient to attract flies (Becher et al. 2012). The ecological

relevance and strength of this attraction is exemplified by the lily, *Arum palaestinum*, whose volatiles mimic yeast fermentation products. The lily volatiles attract flies, which then act as pollinators (Stökl et al. 2010). Several fly odorant receptors (Ors and Irs) detect the smell of yeast (Silbering et al. 2011; Stökl et al. 2010). This complex long-distance detection of, and attraction to, yeast volatiles is consistent with the ecological importance of yeast for flies. But the yeast volatiles alone are not the only chemical cues that attract *D. melanogaster*. The flies are also attracted to some fruit-specific products, such as antioxidants. These fruit antioxidants are hypothesized to help the immune defense of larvae that will eventually hatch on these sites and will have to defend against microorganisms that co-occur with beneficial food yeasts in the flies' habitat (Dweck et al. 2015a). Interestingly, the antioxidants are not sensed directly by the flies. Rather, a yeast metabolite of the antioxidants, the volatile ethylphenol (Figure 2), attracts the flies (Dweck et al. 2015a). Thus, flies are attracted to the combination of yeast and fruit, harking back to the idea that semiochemicals often work as blends, or in this case convey information as a blend, instructing much of a female fly's egg-laying decision.

The chemical cues that are sensed by females are not always attractants. Females can also evaluate chemical cues to avoid sites that are potentially dangerous to their eggs or offspring. For example, *D. melanogaster* females express a sensitive and selective olfactory channel for geosmin (Figure 2), a volatile chemical produced by harmful microorganisms. Detection of geosmin leads to strong repulsion and to avoidance of potential egg-laying (Figure 1g,h; Becher et al. 2010; Becher et al. 2012; Stensmyr et al. 2012). The fly olfactory system therefore is tuned to sensing microorganisms that affect their progeny. Whether these cues are attractant or repellent seems determined by whether they come from microorganisms that are beneficial (mostly yeast) or harmful (e.g. pathogens) (Figure 1g).

Sensing chemical cues associated with nutritional vs. pathogenic features is only one aspect of attraction to an egg-laying site. Other flies can also help. Flies that have already located a food source deposit aggregation pheromones that attract more flies. *Drosophila* females benefit from aggregation through communal egg-laying, which increases offspring survival through cooperation between larvae in fending off fungal growth on the food permitting better resource exploitation (Stamps et al. 2012; Trienens et al. 2017; Wertheim et al. 2002b). The aggregation pheromones that attract females to oviposition sites are complex. The first aggregation pheromone discovered in *Drosophila* (Bartelt et al. 1985) was cVA (Figure 2; Wertheim et al. 2002a; Wertheim et al. 2006). This pheromone was discussed earlier as contributing to decreased mating-attractiveness of mated females when co-detected with 7-T. When co-occurring with the smell of food, cVA functions to attract flies to aggregate (Bartelt et al. 1985; Billeter and Levine 2015; Lebreton et al. 2012; Schlief and Wilson 2007; Xu et al. 2005;), illustrating once again that being in a blend can confer different functions to the same pheromone. cVA is deposited by mated females on egg-laying sites during the process of ejaculate ejection discussed earlier (Figure 1e; Dumenil et al. 2016; Laturney and Billeter 2016). Several other aggregation pheromones that have been subsequently reported include 9-Tricosene (Figure 2), which is deposited by males when sensing the presence of food and attracts females to lay eggs nearby (Lin et al. 2015). Mated females also deposit a series of CH on yeast sources, promoting egg-laying by other mated females (Dumenil et al. 2016). These compounds are deposited through frass (fly excreta),

which contains CH, some cVA, as well as more recently-discovered pheromones such as methyl laurate (Figure 2; Keesey et al. 2016). Aggregation pheromones deposited by *D. melanogaster* are not species-specific, so various *Drosophila* species are regularly found occupying the same oviposition sites. Thus, the benefit of breeding together is likely not restricted to the species level but may benefit closely related species (Symonds and Wertheim 2005; Wertheim 2005). The process of attracting flies to an egg-deposition site has some negative consequences: it can be hijacked by parasitoids. cVA also attracts parasitoid wasps to oviposition sites (Wertheim et al. 2003). These wasps inject their eggs in *Drosophila* larvae, where the eggs then and develop further. In nature, up to 80% of *Drosophila* larvae are parasitized by parasitoid wasps, including *Leptopilina boulardi* and *L. heterotoma* (Fleury et al. 2004).

4b1. Local chemical cues that prompt egg-laying—Once a female has reached a suitable egg-laying substrate, she searches for a site on which to lay her eggs. This searching behavior is triggered by the presence of an ovulated egg in her reproductive tract (Gou et al. 2014). The female walks around, probing the substrate with her legs, proboscis and ovipositor, all of which contain sensory receptors (Yang et al. 2008). When sampling potential oviposition sites, females integrate input from smell and taste to weigh two competing options: egg-laying attraction vs. positional repulsion (Joseph et al. 2009). For instance, females select substrates containing acetic acid (Figure 2) for egg-laying, showing egg-laying attraction, but do not stay on such sites once they have laid the egg, showing positional repulsion. The egg-laying preference for acetic acid is primarily relayed through gustatory neurons, while positional aversion is relayed through the olfactory system (Joseph et al. 2009). Analogous contradictory behaviors in females' responses to a chemical cue are also observed in response to lobeline (Figure 2), an alkaloid that is naturally produced by plants in the genus *Lobelia* (Krochmal et al. 1972), and serves as a feeding repellent for several insect species (Detzel and Wink 1993; Wink and Schneider 1990). The presence of lobeline repels *Drosophila* females from long-stays on a substrate, but attracts these females to lay eggs there (Joseph and Heberlein 2012). The positional repulsion and egg-laying attraction of lobeline is regulated by gustatory neurons: neurons on the tarsi of the female's first pair of legs stimulate positional aversion (Chen and Amrein 2017; Joseph and Heberlein 2012), whereas neurons in the internal mouthparts lining the pharynx receive inputs that stimulate egg-laying attraction (Joseph and Heberlein 2012).

The chemical cues that guide the precise selection of an egg-laying site by females are not directly connected to the cues that attract females to oviposition sites from a long-distance. This is shown by the observation that several chemicals that promote oviposition do not attract flies in olfactory tests. This dichotomy between the mechanisms that determine general site attraction and egg-laying site choice is illustrated by oviposition site choice in the presence of parasitoid wasps. The adult fly's olfactory system is tuned to the *Leptopilina* odor iridomyrmecin, which is a major component of the female wasp sex pheromone (Figure 2; Ebrahim et al. 2015). Flies show no long-range repulsion to the smell of wasps, but female flies will avoid laying eggs when they smell the parasitoid (Figure 1h; Ebrahim et al. 2015; Lefèvre et al. 2012). As *Drosophila* parasitoid wasps do not attack adult flies but only the larvae that hatch from the fly's eggs, it makes sense that female flies are not repelled by

the smell of parasitoids, but merely avoid laying eggs in their presence. The same logic applies to the observation that females prefer to lay egg on citrus fruits (Figure 1i), which produce limonene. Even though limonene does not normally attract flies, its smell repels parasitoid wasps, potentially explaining why flies prefer to oviposit at sites containing limonene (Dweck et al. 2013). Finally, flies avoid laying eggs near feces of carnivorous animals, perhaps because the feces contain harmful bacteria that produce phenol – a volatile detected by and repellent to flies (Mansourian et al. 2016). Egg-laying site preference is thus an chemically-driven behavioral strategy that ensures that offspring are reared in safer environments.

CONCLUSIONS

Chemicals are important agents of communication. In *D. melanogaster,* pheromones include cuticular and other hydrocarbon molecules and seminal proteins from males. These suites of molecules can telegraph the species, maturity, mating-status, and other reproductive characteristics of females to potential mates (of their own or other species). Given the importance of species-specificity in mating on the one hand, and the divergent optimal reproductive strategies of males and females on the other, pheromones show remarkable species specificity at the structural level and in the blends in which they function. Their activity as signals or switches, rather than as downstream effectors of biochemical pathways, is evidence of the broad effect that these molecules have on the fly's biology and evolution. Chemicals that contain information about food substrates and the presence of pathogens or predators, are also sensed by females (and males) including in the selection of optimal sites for egg-laying and progeny development. The valence (attractiveness or repulsiveness) of these latter chemical cues to mated females is connected to whether the species that produce them is beneficial to the female or her progeny, in which case it attracts females, or harmful, in what case it repels them. The complexity of chemical signaling is evident even in the cues made by, or affecting, females – on which we focused this article. The male likely encounters a similar complexity. There is much room for future research to uncover the complexity and expanse of the sensory mechanisms that underlie reproductive behaviors and the details of the chemical mechanisms that couple reproductive success to a fly's intrinsic state, and to ecological and social contexts.

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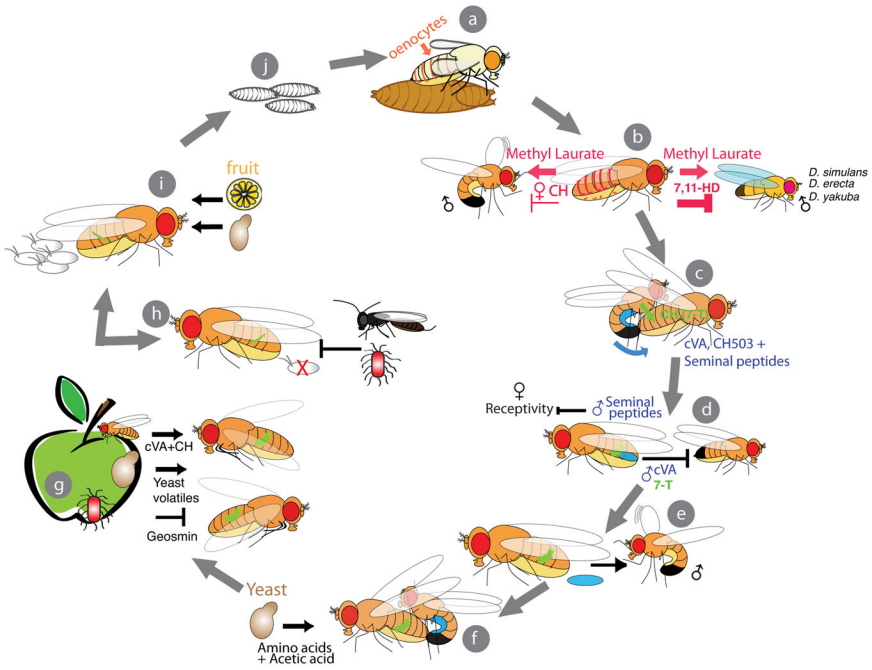

Figure 1. Stages of female reproduction and the chemical cues that guide them

a) newly eclosed females have undeveloped pheromones, which exposes them to forced mating by adult males. The locations of the underdeveloped oenocytes (salmon colored) are indicated by the black arrows. **b)** Mature adult females have developed oenocytes (pink color) that produce some of the chemicals (pink) that attract males from their own species and repel males from other *Drosophila* species. **c)** Males transfer several pheromones to females during mating, including 7-T on the cuticle (green) and cVA, CH503 and seminal peptides in the semen (blue). **d)** These pheromones make females unreceptive and unattractive to males. **e)** Ejection of pheromones from the female's reproductive tract restores some attractiveness. **f)** Mated females that co-detect acetic acid and amino acids from yeast are prone to remating. **g)** Chemical cues from conspecifics, yeast, fruits and pathogens either attract or repel females to fly towards an egg-laying site. **h)** If females sense chemicals produced by harmful species (parasitoid wasps or pathogenic microbes) they will refrain from laying eggs. **i)** If females sense chemicals from beneficial species they will lay eggs. **j)** Eggs will hatch into larvae that will eventually pupate and eclose as adults, completing the reproductive and chemical cycle.

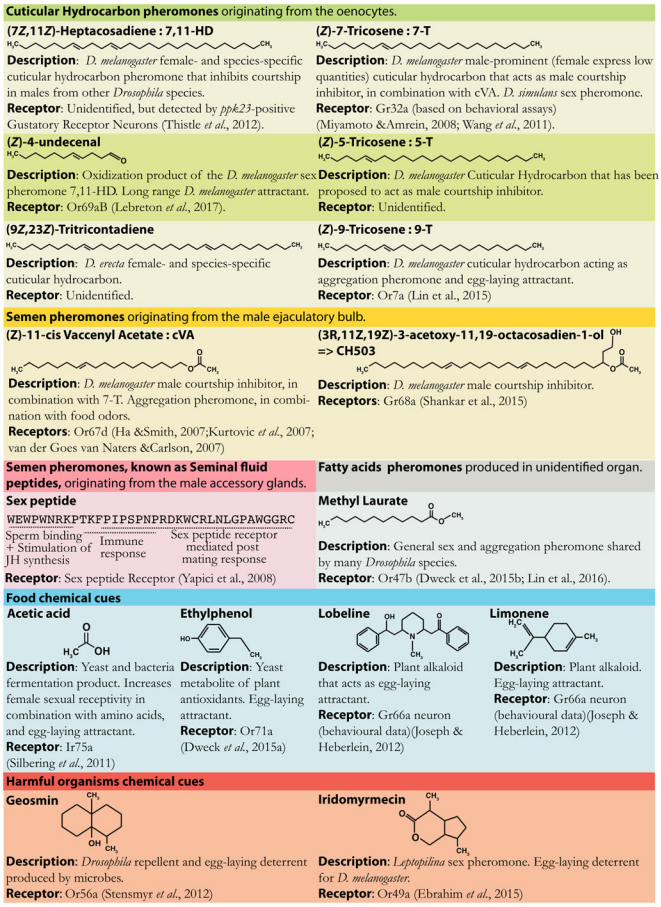

Figure 2. Structure of selected molecules that influence *D. melanogaster* female reproductive behaviors

Amino acid regions of the Sex peptide that have been associated with distinct functions (Kubli, 2008) are underlined. Sequences of other seminal pheromones (such as the 264-aa ovulin) are not shown in this figure.