How resources control aggression in *Drosophila*

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ABSTRACT

How animals use sensory information to weigh the risks vs. benefits of behavioral decisions remains poorly understood. Inter-male aggression is triggered when animals perceive both the presence of an appetitive resource, such as food or females, and of competing conspecific males. How such signals are detected and integrated to control the decision to fight is not clear. Here we use the vinegar fly, *Drosophila melanogaster,* to investigate the manner in which food and females promotes aggression.

In the first chapter, we explore how food controls aggression. As in many other species, food promotes aggression in flies, but it is not clear whether food increases aggression per se, or whether aggression is a secondary consequence of increased social interactions caused by aggregation of flies on food. Furthermore, nothing is known about how animals evaluate the quality and quantity of food in the context of competition. We show that food promotes aggression independently of any effect to increase the frequency of contact between males. Food increases aggression but not courtship between males, suggesting that the effect of food on aggression is specific. Next, we show that flies tune the level of aggression according to absolute amount of food rather than other parameters, such as area or concentration of food. Sucrose, a sugar molecule present in many fruits, is sufficient to promote aggression, and detection of sugar via gustatory receptor neurons is necessary for food-promoted aggression. Furthermore, we show that while food is necessary for aggression, too

much food decreases aggression. Finally, we show that flies exhibit strategies consistent with a territorial strategy. These data suggest that flies use sweet-sensing gustatory information to guide their decision to fight over a limited quantity of a food resource.

Following up on the findings of the first chapter, we asked how the presence of a conspecific female resource promotes male-male aggression. In the absence of food, group-housed male flies, who normally do not fight even in the presence of food, fight in the presence of females. Unlike food, the presence of females strongly influences proximity between flies. Nevertheless, as group-housed flies do not fight even when they are in small chambers, it is unlikely that the presence of female indirectly increases aggression by first increasing proximity. Unlike food, the presence of females also leads to large increases in locomotion and in male-female courtship behaviors, suggesting that females may influence aggression as well as general arousal. Female cuticular hydrocarbons are required for this effect, as females that do not produce CH pheromones are unable to promote male-male aggression. In particular, 7,11-HD—a female-specific cuticular hydrocarbon pheromone critical for male-female courtship—is sufficient to mediate this effect when it is perfumed onto pheromone-deficient females or males. Recent studies showed that *ppk23*$^{+}$ GRNs label two population of GRNs, one of which detects male cuticular hydrocarbons and another labeled by *ppk23* and *ppk25*, which detects female cuticular hydrocarbons. I show that in particular, both of these GRNs control aggression, presumably via detection of female or male

pheromones. To further investigate the ways in which these two classes of GRNs control aggression, I developed new genetic tools to independently test the male- and female-sensing GRNs. I show that *ppk25-LexA* and *ppk25-GAL80* faithfully recapitulate the expression pattern of *ppk25-GAL4* and label a subset of *ppk23*$^{+}$ GRNs. These tools can be used in future studies to dissect the respective functions of male-sensing and female-sensing GRNs in male social behaviors.

Finally, in the last chapter, I discuss quantitative approaches to describe how varying quantities of food and females could control the level of aggression. Flies show an inverse-U shaped aggressive response to varying quantities of food and a flat aggressive response to varying quantities of females. I show how two simple game theoretic models, “prisoner’s dilemma” and “coordination game” could be used to describe the level of aggression we observe. These results suggest that flies may use strategic decision-making, using simple comparisons of costs and benefits.

In conclusion, male-male aggression in *Drosophila* is controlled by simple gustatory cues from food and females, which are detected by gustatory receptor neurons. Different quantities of resource cues lead to different levels of aggression, and flies show putative territorial behavior, suggesting that fly aggression is a highly strategic adaptive behavior. How these resource cues are integrated with male pheromone cues and give rise to this complex behavior is an interesting subject, which should keep researchers busy in the coming years.

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INTRODUCTION

In this introduction, I will review the existing literature on how sensory cues control intraspecific social behaviors in vertebrate and invertebrate species with a special focus on aggression. The sensory cues relevant for aggression can be broadly divided into two categories: opponent signals and resource signals. I will identify examples of these two categories of signals in multiple organisms and their effects on behavior. Then, I will discuss a few conceptual missing pieces in the literature as they pertain to control of aggression, which serves as the foundation for the thesis. Other important topics relevant to aggression, such as how sensory information described below converges in the central nervous system to integrate different sensory inputs, and how the internal states of the animal, such as hunger, reproductive drive, and social isolation modulate these processes, will not be covered.

Metazoan organisms in nature constantly face behavioral choices. How animals use sensory information to weigh the risks vs. benefits of behavioral decisions remains poorly understood. Aggression is an instinctive social behavior found in all metazoan species including flies, mice and humans. It is an ideal system to study how the nervous system makes value-based decisions, as the decision to fight comes with apparent cost and benefits and requires the assessment of a potential conflict: the detection of attractive resources and competitors who limit access to such resources. Although much focus has been given to how male-specific signals control aggression in model organisms such as mice and flies, much less is known about how resource signals contribute to

aggression. Identification of resource-specific cues and the neural circuits, which process them, are essential steps to understanding how a complex behavior is regulated by the integration of multiple inputs.

Gender-specific signals relevant to aggression

Proper recognition of gender is critical to the survival of species. Among species that display territorial behaviors, the choice between the execution of courtship behavior vs. aggressive behavior depends on the proper identification of the gender. There is large variation among different species on the type of signals used for gender recognition. Gender-specific cues used to advertise and recognize conspecific competitors across phyla can range from chemicals, auditory cues, visual cues, or behavioral patterns (Grether, 2011). Males use these signals to both advertise their presence intentionally and to detect opponents in the context of defending resources (Baker, 1983). In some animals, these signals promote aggression, while in others, detection of these signals is enough for the intruders to move on (Baker, 1983). It has been known for a long time that the conspecific and gender recognition require multiple sensory systems (Partan and Marler, 2005; Tinbergen, 1951; 1959), but recent advances in molecular neuroscience have identified some specific cues and the neural circuits that process them. Identification of gender-specific cues provides a critical entry point to understanding how sensory cues are integrated to give rise to perception of a conspecific mate or rival, and ultimately, the execution of proper social behaviors.

Humans

It is unclear how gender-specific signals guide social behaviors such as aggression in humans, as inter-male competition in humans takes many forms, and these behaviors are not amenable to experimental studies for ethical reasons. Nevertheless, it is likely that gender-specific signals guide appropriate social response in humans as in many other species.

Although human social behaviors may not be stimulus-dependent in the way a mouse or a fly's social behaviors are subservient to pheromones, sensory impairment, such as loss of vision, significantly impairs social behaviors in humans (Dodge, 1979; Kef and Bos, 2006). Furthermore, while individual cues that signal the presence of a male or a female (e.g. a male face or a female voice) may serve redundant functions, brain-imaging studies suggest that these cues may ultimately converge into common neural circuits, which represent a conspecific male or a female (Mouchetant-Rostaing et al., 2000).

Humans are able to discriminate between genders and individual identities using many different cues. This is achieved, in part, by visual and auditory cues and to a lesser extent, chemical cues. According to a poll, human subjects rate physical attractiveness among the most important factors in mate selection (Buss and Schmitt, 1993), and it is often stated in popular culture that humans are "visual animals." This is, at least in part, based on physiological evidence, as much of the human brain is devoted to visual processing, and humans lack exquisite

chemosensory discrimination abilities observed in other mammals. For instance, humans have fewer than 350 intact genes encoding odorant receptors (ORs), while rodents have many more than 1000 (Liberles, 2014; Quignon et al., 2005). In addition, while dogs have 230 million olfactory receptor neurons, humans only have 10 million (Kohl et al., 2001). Humans also lack gross anatomical structures, such as vomeronasal organs (VNO) and the accessory olfactory bulbs (AOB), which are used to detect pheromones in amphibians, reptiles and nonprimate mammals (Keverne, 1999). Consequently, humans seem to rely heavily on visual cues to distinguish between genders and individuals.

Humans are exquisitely sensitive to gender-specific visual cues. Human males and females have gross anatomical differences, such as height, body shape, and primary sexual organs. However, in addition to these readily identifiable visual cues, humans can discriminate genders apart from more abstract visual cues, such as gait patterns (Kozlowski and Cutting, 1977) and subtle facial features (Bruce et al., 1993). Facial features, in addition to conveying information about gender, also carry additional social cues such as threat display or appeasement (van Staaden et al., 2011).

In addition to visual sexual dimorphism, human males and females have different voice pitch (Ardila, 1993), which can be used to discriminate genders (Bachorowski and Owren, 1999; Gaetano et al., 2014). In addition, vocal pitch height can be used to convey mood and social hierarchical information, such as submissive or aggressive attitude (Greenberg et al., 1978).

The existence of human pheromones is a somewhat controversial subject (Liberles, 2014). As mentioned above, humans lack gross anatomical structures (VNO, AOB) used in other species for pheromone detection. Furthermore, the human genome either does not encode or has non-functional versions of genes such as *TRPC2, V1R, V2R*, *MUPs,* and *ESPs*, which are genes encoding pheromone receptors and protein pheromones in rodents (Liberles, 2014). Nevertheless, there are studies that demonstrate the evidence of chemical communication in humans (Keller et al., 2007; Zhou et al., 2014). For instance, the menstrual cycle of one individual can be regulated by body-derived chemicals of another (Stern and McClintock, 1998), and gender-specific chemicals such as androstadienone and estratetraenol have been shown to modify emotional states and gender perception in a sexually dimorphic manner (Jacob et al., 2001; Zhou et al., 2014). Furthermore, components present in female tears can decrease male sex drive (Gelstein et al., 2011). It remains unclear how these gender-specific chemicals are detected in humans; however, in principle, the main olfactory epithelium (MOE) could be used as in mice (Liberles, 2014), to detect pheromone-like chemicals, possibly via currently unidentified pheromone receptors.

Non-human primates

Like humans, non-human primates use multisensory cues to distinguish not only conspecifics from other species, but also genders and individual identities among them. Similar to humans, non-human primates primarily use visual and

auditory cues to distinguish the genders of conspecifics. In monkeys, such as mandrills, (*Mandrillus sphinx*), males display conspicuous secondary sexual traits such as red skin color and darkened testicles to convey information about gender and rank within the hierarchy (Gerald, 2001). Interestingly, these visual cues undergo changes in a reversible manner as the monkey's rank rises and falls (Gerald, 2001). Presence of dark scrota is sufficient to reduce aggression from opponent males, and this effect can be mimicked by a paint treatment of a non-dominant male, suggesting that this effect is entirely visual (Gerald, 2001).. Visual recognition of conspecific males seems to be, at least in part, innately conditioned: socially-isolated 9 month-old monkeys (*Macaca mulatta)* react with particular saliency to threatening pictures of conspecific males (Sackett, 1966). In addition to visual cues, non-human primates also use auditory cues to identify gender and individual identity (Ghazanfar and Santos, 2004). Vocalizations have individual variations, and they are used to convey information ranging from body size, reproductive status, group membership and dominance.

Although there is some experimental evidence supporting the existence of pheromones in non-human primates, the extent to which they contribute to sexually dimorphic behaviors is unclear (reviewed in [Grammer et al., 2005]). Similar to humans, non-human primates lack functional VNOs, and many of the genes required pheromone detection in rodents do not appear to be functional in primates (Liman and Innan, 2003; Zhang and Webb, 2003). Nevertheless, there is some experimental evidence of various gender-specific chemicals exerting their

influence on sexual behaviors. For instance, chemicals that are indicative of ovulating females, such as aliphatic acids in vaginal secretions that are also present in humans, have been shown to induce sexual arousal in primates (Curtis et al., 1971; Michael and Keverne, 1968; Michael et al., 1974). Like in humans, these studies demonstrate that there may be sexual communication via pheromones, but the molecular identity and the mechanism by which they are detected and modify behaviors such as aggression and mating remains elusive.

Mice

Mice are social creatures, and they are able to discriminate species, gender, and individual identity using mainly chemical cues (Liberles, 2014). Although both auditory (Chabout et al., 2012; Holy and Guo, 2005) and visual cues can influence social behaviors in mice (Jones and Nowell, 1973), they appear to play minor roles compared to olfactory cues (Hedrich, 2004; Scott and Fredericson, 1951; Van Loo et al., 2003). As such, most of the focus on mouse social behaviors has been on the identity of pheromones and sensory mechanisms detecting them.

There are many physiological sources of gender-specific pheromones in mice, such as sweat, saliva, urine, etc. Urine contains some aggression-promoting volatile chemicals (Novotny et al., 1985) and proteins (Chamero et al., 2007), which promote male-male aggression. In addition to these chemicals, there are other classes of gender-specific pheromones, such as steroid derivatives, analogous to androstenone found in male humans, and exocrine gland-secreting

peptides (ESPs), but these do not appear to function in male-male aggression.

As mentioned above, VNO plays an important role in pheromone detection in many species, including mice. VNO is responsible for detecting pheromones from multiple physiological sources, such as urine, tears, and saliva (Liberles, 2014). Within the VNO resides VNO sensory neurons, whose function requires families of odorant receptors used to detect pheromones: vomeronasal receptors type 1, type 2 (V1R and V2R), and formyl peptide receptors (FPR) (Liberles, 2014). Mice with disrupted VNO function, either by surgical or genetic manipulations, display aberrant social behaviors, including aggression (Chamero et al., 2011; Stowers, 2002; Wysocki and Lepri, 1991). In addition to the VNO, the MOE is also known to play a role in aggression (Mandiyan et al., 2005).

Other vertebrate species

Various species of birds are used in studies of aggression, as birds of many species exhibit inter-male aggression (Grether, 2011). Males of many bird species establish territories, which they defend seasonally or throughout the year. Birds lack VNO, and there is little to no evidence of pheromonal communication (Keverne, 1999). Instead, birds use both of these auditory cues and visual cues to identify conspecific opponents, although there is variation among bird species as far as the extent to which one modality is used vs. another (Grether, 2011). Male birds of many species signal to each other via threat displays (Hurd and Enquist, 2001) and vocalizations (i.e., bird calls and bird songs) to communicate with each

other (Avey et al., 2011; Nowicki et al., 1998). These signals are necessary for males to defend their territory adequately, and presentation of artificial visual or auditory cues produces behavioral outputs in opponents (Nowicki et al., 1998; Peek, 1972).

Like birds, reptiles and amphibian species are also often used for studies of intraspecific aggressive behaviors. Skinks (lizards) distinguish conspecific males from either females or other species by using sexually dimorphic visual and chemical cues (Cooper and Vitt, 1987; 1988). Male skinks act aggressively toward females painted with male-specific orange colors on their heads, but stop once they tongue-flick the females, suggesting that they may rely on visual cues from a large distance but more on chemical cues in short distances (Cooper and Vitt, 1988). Other lizards (*E. inexpectatus* [Cooper and Vitt, 1987] and *Podarcis hispanicus* [L pez et al., 2002]) also rely on chemical cues to recognize conspecific males.

Invertebrate species (excluding Drosophila melanogaster)

Cephalopods, which include squid and octopus, have complex nervous systems, and accordingly display a complex array of social behaviors. They rely heavily on visual communication and use skin color and posture to convey gender information (reviewed in [van Staaden et al., 2011]).

Spiders are known to engage in complex social behaviors. In particular, jumping spiders, *Thiania bhamoensis*, also known as “fighting spiders,” have acute

vision due to their large eyes (Li et al., 2002). True to their name, male spiders display complex ritualized agonistic interactions, with fifteen documented steps of engagement, including leg-raising and leg-shaking while walking sideways before striking (Li et al., 2002). Male spiders of this species are blue, while females are green, and male spiders are able to distinguish the gender of conspecifics without touching them (Li et al., 2002).

Crickets, like *Drosophila melanogaster*, are non-social insects, whose males engage in ritualized fighting behavior (Kravitz and Huber, 2003). Crickets are able to determine the gender of the conspecific member by detecting gender-specific cuticular hydrocarbon (CH) pheromones, and engage in courtship or aggression depending on the CH cues (Brown et al., 2006; Iwasaki and Katagiri, 2008; Tregenza and Wedell, 1997). Visual cues modulate fighting behavior in crickets, but the effects are mainly to suppress fighting, rather than to enhance them (Rillich et al., 2007). Visual cues such as size of the opponent allow for proper assessment of possible outcome of the agonistic encounters, and without them, smaller crickets do not flee when faced with a bigger opponent (Rillich et al., 2007).

Drosophila melanogaster

Drosophila males fight other males and court females. They accomplish this behavioral specificity by using multiple sensory modalities, including visual, auditory, olfactory and gustatory cues.

Vision is important to proper male social behaviors (Krstic et al., 2009; 2013). In the dark, males court females less (in bigger chambers) and court males more (Krstic et al., 2009). Visually-impaired mutants, such as *ninaB*^{360d} and *w*^{1118}, court less than wildtype flies (Krstic et al., 2013). Visual cues do not specify gender recognition per se, since flies indiscriminately court moving objects, whether they are males or even another species (Yamamoto and Koganezawa, 2013). Visual signals also contribute to male-male aggression, as flies do not fight in the dark, and visually impaired mutant flies such as *norpA*^{P24} and *ninaE*^{17} flies do not fight (Hoyer et al., 2008). Furthermore, males are able to detect the presence of other flies of both genders, and change their mating duration accordingly (Kim et al., 2012). This effect is dependent upon movement of red compound eyes, since moving females or their own reflection in the mirror are able to reproduce this effect, but not white-eyed flies (Kim et al., 2012). Although mutations that affect vision such as *white* and *ninaB* have pleiotropic effects outside the visual system (Halme et al., 2010; Hoyer et al., 2008; Oxenkrug, 2010), these results suggest that visual cues play an important role in detecting the presence of another fly and modulate social behaviors.

Auditory cues also play an important role in social behaviors in fruit flies, although it is unclear whether they have a functional role in aggression. Males court females by vibrating their wings to produce a courtship song, which primes females for copulation and enhances mating success (Kyriacou and Hall, 1982). Sound production during aggressive encounters has also been recorded, but it is

unclear whether it has functional consequence, since visual cues from aggressive movements such as wing threats can produce sounds (Jonsson et al., 2011).

Like in many other insect species, sex-specific chemosensory cues play a dominant role in *Drosophila* social behaviors. Male and female flies have different pheromone profiles (Billeter et al., 2009; Ferveur et al., 1997; Jallon, 1984), and manipulations of these gender-specific pheromones affect courtship and aggression (Fernández and Kravitz, 2013; Wang et al., 2011). Among male-specific volatile pheromones, *cis*-11- vaccenyl acetate (cVA), a male-specific pheromone found in the male ejaculatory bulb, controls aggregation (Bartelt et al., 1985), courtship (Zawistowski and Richmond, 1986) and aggression (Wang and Anderson, 2010). Flies detect cVA via Or67d-expressing olfactory receptor neuron (*Or67d*$^{+}$ ORNs) and *Or65a*$^{+}$ ORNs, which are found in the trichoid sensilla on the *Drosophila* antennae (Clyne et al., 1997; Ha and Smith, 2006; Kurtovic et al., 2007; Liu et al., 2011; van der Goes van Naters and Carlson, 2007; Xu et al., 2005). *Or67d*$^{+}$ ORNs acts acutely to increase aggression (Wang and Anderson, 2010), while *Or65a*$^{+}$ ORNs reduce aggression via chronic exposure to cVA (Liu et al., 2011). In addition to cVA, other ORNs such as *Or47b*$^{+}$ ORNs (Lone and Sharma, 2012; van der Goes van Naters and Carlson, 2007; Wang et al., 2011), and *Or88a* (van der Goes van Naters and Carlson, 2007) also participate in detecting fly odors, although they have not been implicated in aggression.

Gustatory cues seem to be particularly important for social behaviors, as they are indispensable for gender-recognition in flies, unlike olfactory cues (Wang and Anderson, 2010). Males whose CH profiles are feminized or abolished genetically elicit courtship despite appearing male (Billeter et al., 2009; Ferveur et al., 1997; Wang et al., 2011), and females whose CH profiles are masculinized elicit male aggression from males (Fernández and Kravitz, 2013). Among numerous male-specific CHs, 7-tricosene (7-T) is sufficient to restore aggression from other males when painted on pheromone-blank (oe-) males (Wang et al., 2011).

The ecological function of 7-T and the mechanism of 7-T detection is complicated. In addition to 7-T's role in male-male aggression, 7-T also decreases male-male courtship in a *Gr32a*- and *Or47b*- dependent manner (Wang et al., 2011). Furthermore, 7-T and *Gr32a* also inhibit interspecies mating in *Drosophila melanogaster* males. Interestingly, in addition to 7-T, 9-T (*z*-9-tricosene) and 11-P (*z*-11-pentacosene), which are present in other drosohphilids, inhibit courtship in *D. melanogaster* males in a *Gr32a*-dependent manner (Fan et al., 2013). *Gr32a*'s role in promoting male-male aggression and inhibiting male-male courtship seems to be, in part, due to 7-T detection (Wang et al., 2011). 7-T response is seen in bitter-sensing (*Gr66a*$^{+}$) GRNs and octopaminergic neurons in the brain in *Gr32a*$^{+}$ GRN-dependent manner (Andrews et al., 2014; Inoshita et al., 2011).

In addition to *Gr32a*$^{+}$ GRNs, a distinct (albeit partially overlapping)

population of GRNs—*ppk23*$^{+}$/*fruitless*$^{+}$ GRNs—also play a role in male CH detection (Thistle et al., 2012). *ppk23*$^{+}$ GRNs show response to male pheromones, including 7-T, 7-P and cVA (Thistle et al., 2012). Importantly, the Calcium imaging response to male pheromones in *ppk23*$^{+}$ GRNs was seen in the tarsal cell bodies and labellar cell bodies using a UAS-GCaMP3, while the Calcium response to 7-T in *Gr66a*$^{+}$ GRNs was seen in the SOG (more than 10 minutes after the stimulus delivery) using a less well-characterized UAS-GFP-Aequorin (Inoshita et al., 2011). Direct comparisons of these two experiments are difficult, as authors of each study only characterized either the *ppk23*$^{+}$ or *Gr66a*$^{+}$ GRNs. In addition to the response to male pheromones, functional manipulations of *ppk23*$^{+}$ GRNs showed that *ppk23*$^{+}$ GRNs normally play a role in detecting male pheromones to decrease male-male courtship in a bitter-sensing *Gr66a*-independent manner (Thistle et al., 2012).

As mentioned above, *ppk23*$^{+}$ GRNs and *Gr32a*$^{+}$/*Gr66a*$^{+}$ GRNs have partial overlap (in the proboscis), which may explain the redundant function of *ppk23*$^{+}$ GRNs and *Gr32a*$^{+}$ GRNs in male-male courtship. Interestingly, Fan *et al.* showed that only *Gr32a*$^{+}$ GRNs, but not *Gr66a*$^{+}$ or *ppk23*$^{+}$ GRNs, function to inhibit interspecies mating, suggesting that *Gr32a*$^{+}$ GRNs may have different function from both ppk23+ GRNs and *Gr66a*$^{+}$ GRNs. At present, it is unclear which of these GRNs function in detecting 7-T and other male-specific CHs to promote male-male aggression. The preliminary data in Chapter 3 of this thesis suggest that, like *Gr32a*$^{+}$ GRNs, *ppk23*$^{+}$ GRNs are also necessary for male-male

aggression.

Resource signals relevant to aggression

Access to resources is necessary for survival, and almost all forms of intraspecific aggression is related to resources. Despite its importance, the contribution of resources to aggression is rarely the focus of studies in many species (Janson and van Schaik, 1988). Thus, in the fields of ethology or neuroscience, resource-mediated control of aggression is often treated as a given, with some variation of the following sentence offered in the introductions: '(the species in the study) fight over resources, such as food or females' (Egge et al., 2010; Potter and Luo, 2008). Studies that focus on resource's contribution to aggression usually come from the fields of evolutionary biology and ecology, where it is observed that most animals seem to compete over resources.

So, which resources do animals fight over? Females are a common source of competition among males of many species, as will be discussed below. Food provides another resource over which to compete. In many non-territorial species, conspecifics congregate on common food resources, where they carry out most social activities, including aggression and reproduction (Brown, 1970). In territorial species, conspecifics may defend territories containing food and home areas. Still in others, males compete over mating territories, called leks, which can range from being as specific as rotting leaves, on which oviposition occurs, to nonspecific areas without food (Shelly, 1987).

Although species-specific differences exist, since resource controls aggression in many organisms (the evidence of which will be presented below), it is likely that there are basic principles governing resource-control of aggression, which are conserved through evolution. In this section, I will lay out examples among selected species where food and female cues or the presence of a “home” territory controls male-male aggression.

Humans

From the mythical accounts of Helen of Troy to modern warfare over valuable commodities or territory, it should be intuitively obvious that humans compete for resources. Nevertheless, studies on human aggression tend to put more emphasis on human-specific factors: internal variables such as emotional control or lack thereof, and external variables such as use of drugs and alcohol (Anderson and Bushman, 2002). I will present the evidence that shows that human aggression is also influenced by basic resources such as females, food, water and territories.

Meta-analyses of available historical data across many cultures suggest that resource unpredictability (Ember and Ember, 1992), caused by environmental stressors and climate change (Hsiang et al., 2013), can account for most records of human warfare. In primitive societies, human groups engaged in warfare over females or material wealth (Manson et al., 1991). In groups where material wealth exists and is transferrable (Northwest Coast Indians), intergroup aggression tends to revolve around material wealth (Manson et al., 1991). In contrast, in groups of

foragers, where material wealth is usually not alienable (Eskimos, Australian Aborigines), females are the main cause of intergroup aggression (Manson et al., 1991). In more contemporary Western settings, social scientists have proposed that the institution of marriage is functionally equivalent to mate-guarding (Bethmann and Kvasnicka, 2010), which is a form of reproductive competition observed in many different species across phyla. Although aggression takes many subtle forms in humans other than outright violent individual or group competitions, sexual jealousy (Buss, 2002) and stalking (Duntley and Buss, 2010) are both *competitive* strategies employed by males to ensure exclusive mating. According to polls, sexual infidelity among committed partners is rather common, and it is thought that these strategies allow males to prevent access of their partners by potential poachers and avoid cuckoldry (Buss, 2002).

As stated above, in societies with developed economies, humans also compete over material wealth. Violent crimes rise and fall with economic environments within societies (Archer, 2009a; 2009b). Poverty and income inequality lead to higher incidences of violent crimes, both when compared across cultures and when compared within the same culture across different times (Fajnzlber et al., 2002; Hiraiwa-Hasegawa, 2005; Hsieh and Pugh, 1993; Kennedy et al., 1998). In particular, income inequality, which is often measured by the Gini coefficient, seems to be an important factor in driving violent crimes (Fajnzlber et al., 2002).

On an individual level, humans also act more aggressively when there is a

perceived lack of food (Stucke and Baumeister, 2006). Furthermore, another study demonstrated that hunger, measured by low blood glucose concentration, can correlate with increased aggressive actions in humans (Bushman et al., 2014). Other studies have confirmed the relationship between sugar and aggression, as glucose consumption reduces aggression and improves self-control (Denson et al., 2010; Gailliot et al., 2007; Hagger and Chatzisarantis, 2012). Furthermore, diseases that affect glucose metabolism, such as diabetes, increases aggression in human subjects (DeWall et al., 2011). It is particularly interesting to note that sugars have been shown to control aggression in rats (Lore et al., 1986) and in insect species such as (Johnson and Hubbell, 1974) and *Drosophia melanogaster,* although in these cases, sugar increases aggression, suggesting a possible mechanistic link between human aggression and aggression in other species.

Non-human Primates

Like humans, primates also compete over many different resources, including females (Alberts et al., 1996; Watts, 1998), food (Janson, 1985) (reviewed in [Janson and van Schaik, 1988]) and home territories (Mitani et al., 2010). Females are transferred in some primate species (chimpanzees), while in others (Vervet monkeys, savannah baboons, wedge-capped Capuchins), females stay within their home group (philopatry) (Manson et al., 1991). When females are transferable, as seen in chimpanzee groups, the main source of aggression was

over females (Manson et al., 1991). In such cases, females who are sexually receptive are spared, while those who are not, or are older, are killed (Williams et al., 2004). In contrast, among primate species where females exhibit philopatry, groups only compete over food resources such as fruiting trees, water, and territory with these resources (Manson et al., 1991). This is not to suggest, however, that chimpanzees do not fight over other resources. In fact, chimpanzees are known to compete over territory, and will kill other chimpanzees to protect their group's territorial boundaries and expand their territories through killing (Mitani et al., 2010).

Like humans, non-human primates also exhibit more subtle forms of competitive strategies, such as mate-guarding. For instance, chimpanzees are known to guard their mates, even cooperatively among a group of males who share access to the same females (Watts, 1998). In addition, male baboons (*Papio cynocephalus*) guard their mates, and interestingly, those who spend more time guarding their mates move more and eat less, suggesting that males are willing to guard their mates at a cost (Alberts et al., 1996).

Rodents

Mice and rats display aggressive behaviors over food, and increase aggression when they are starved (Scott and Fredericson, 1951). In addition to food, both male and female mice fight more in the presence of a water resource (Gray et al., 2002) and territory enclosing their nests, often with lactating females

(Scott, 1966). There are some differences in resource-driven aggression in mice and rats, which are unlike other animals: 1) Mice and rats also display high levels of aggression in the absence of food, and fights over food are shorter, less intense and qualitatively different from when they are for the purpose of "injuring or escaping from the opponent" (Scott and Fredericson, 1951). 2) Mice and rats do not seem to fight over females, which have the effect of *decreasing* aggression in some cases (Scott, 1966).

In other rodents, such as prairie voles (Stehn et al., 1976) and squirrels (Sherman, 1989), females increase male-male aggression. Furthermore, the presence of female odors present in urine from estrous females is sufficient to increase male-male aggression in a context-dependent manner, such as previous mating experience (Stehn et al., 1976). It is unclear whether the seeming lack of female-promoted aggression and low aggression over food in some rodents is due to specific experimental context (such as the type of females used, prior mating experience of males, etc), or due to species-specific differences. It is possible that mice and rats, which do not exhibit mate-based pair bonding (Donaldson and Young, 2008) as in prairie voles, also show their apparent apathy toward the opposite sex by choosing not to fight over females. It is also possible that defending other resources such as food, water, and nesting territory is sufficient to confer mice and rats with access to females. Nevertheless, it has been shown that the presence of estrous females increases plasma testosterone in males (Koolhaas et al., 1980), and testosterone is implicated in aggression in many

systems (Anholt and Mackay, 2012); thus, it is possible that there are contexts in which mice and rats may also compete over females.

Other vertebrate species

As mentioned in the previous section, many species of birds display aggression (reviewed in [Maney and Goodson, 2011]). During mating seasons, migratory male birds establish territories containing food sources and nest sites, and defend them (Maney and Goodson, 2011). In many lekking avian species, many males congregate on mating territories (i.e., leks), where they compete over females who visit them, ostensibly, just for reproduction (Baker, 1983; Beehler, 1983). Depending on their success in territorial defense and the quality of the territories, male birds attract female birds (Maney and Goodson, 2011). Many studies show that birds of many species and throughout developmental stages fight over food. Seabirds compete over food in mating seasons (Furness and Birkhead, 1984; Lewis et al., 2001). Honeyeaters (*Lichenostomus* and *Melithreptus*) fight more in the presence of sites with enriched food sources (Mac Nally and Timewell, 2005). In some bird species (Blue-footed Booby, Black-legged Kittiwake, Osprey, etc), broodmates are known to fight and even kill each other for food (Drummond, 2001). In addition to food and territories containing them, male songbirds also display overt aggression in the presence of females (Goodson et al., 2009).

Other vertebrate species across phyla, from large to small, also fight over

food or females. Male elephants fight over females, and the males who guard more females successfully are able to copulate more (Poole, 1989). Male elephant seals, among whom only less than one-third mate at all, also fight over females (Le Boeuf, 1974). Among reptile species, lizards are also known to compete over both food and females (*Anolis aeneus*) (Pafilis et al., 2009; Stamps, 1977). Salamanders (Gabor and Jaeger, 1995) also compete over food resources, and so do snakes (*Oligodon formosanus*) (Huang et al., 2011).

Many different species among fish have also been observed to fight in the presence of food and females: Coho salmon, Brown Trout, White Seabream, Convict Cichlid, Japanese Medaka Ruffe, Blue Gourami, and Zebrafish are all known to fight over food (Ward et al., 2006), and Beaugregory damselfish are known to fight over females (Santangelo et al., 2002).

Invertebrates

Many invertebrate species compete over resources. Crustaceans guard their mates before mating, even against the wishes of females who become hostile (Jormalainen, 1998). Other invertebrates in the ocean, such as male octopuses, fight over females (Huffard et al., 2010). Squids, in particular, fight over females (DiMarco and Hanlon, 2010), and specific female-derived pheromone, β-MSP, have been has been shown to increase aggression (Cummins et al., 2011). Similarly, spiders that also fight over females (Austad, 1983; Rypstra et al., 2009; Wise, 2006) have been shown to increase fighting in the presence of chemical as

well as visual cues of females (Rypstra et al., 2009).

Among insects, cockroaches (*Gromphadorhina portentosa*) fight more in the presence of females (Guerra and Mason, 2005), and so do spider mites (*Tetranychus urticae*) (Potter et al., 1976). Male crickets, whose aggressive behaviors are well-characterized, also fight in the presence of food (Nosil, 2002) and females (Tachon et al., 1999). Crickets, like many other insects, use cuticular hydrocarbon pheromones for sex recognition (Nagamoto et al., 2005), and female pheromones have been shown to be sufficient to increase male-male aggression (*A. domesticus*) (Otte and Cade, 1976). Winning fights has clear consequences in many insect species, and in crickets, females have been shown to prefer pheromones from winning male crickets, although it is not clear whether this is due to different chemical composition or amount (Kortet and Hedrick, 2005). Finally, stingless bees, which have been shown to fight over food, fight more in the presence of increasing concentrations of sucrose (Johnson and Hubbell, 1974).

Flies (excluding Drosophila melanogaster)

As it has been discussed above in many species, acquisition of food and territories containing food is inherently linked to acquisition of mates for reproduction. In one fly species, *Calopteryx splendens xanthostoma*, it was shown that males who gain access to food by winning fights mate a remarkable 1000 times more often than those who do not (Plaistow and Siva-Jothy, 1996). Accordingly, many fly species display aggressive behaviors over territories that

contain food. *Drosophila conformis* fight over food and territory (which are leaves for this species) (Shelly, 1987). *Dryomyza anilis* fight over food (small carcasses) and females (Otronen, 1984). *Drosophila sechelia*, which exclusively feed on a host plant called Tahitian Noni (*Morinda citrifolia*) (Jones, 2005), fight over noni juice (personal communications, Kenta Asahina).

Male flies of many species also fight over females. Some species display mate-guarding strategies by using mating plugs, mate grasping, or mate monitoring (Alcock, 1994). *Drosophila hibisci* use mating plugs after copulation in order to prevent multiple mating by females (Polak et al., 1998). Crane fly species guard their mates after copulation during female oviposition to ward off other males (Adler and Adler, 1991). In Mediterranean fruit flies, males who mate longer has a higher success rate of sperm transfer (Taylor and Yuval, 1999).

In some *Drosophilid* species on Hawaiian islands, such as *Drosophila conformis* (Shelly, 1987), *D. mycetophaga* (Aspi and Hoffmann, 1998), *D. crucigera* and *D. grimshawi* (Spieth, 1974), lekking behavior is observed. In these species, males occupy leks, which they defend against intruders and advertise toward females (Aspi and Hoffmann, 1998; Spieth, 1974). Males act aggressively toward each other in order to get more desirable leks, which are more frequently visited by females (Shelly, 1987).

Drosophila melanogaster

Drosophila melanogaster males seem to fight over both females and food

(Chen et al., 2002; Hoffmann and Cacoyianni, 1990; Skrzipek et al., 1979; Yuan et al., 2014), although it is unclear from these studies whether males are fighting over food or females, as they are always presented together. Interestingly, males who are continuously exposed to females for 24 hours no longer fight over females, suggesting that males can be conditioned to not fight in the presence of females (Yuan et al., 2014). This effect is independent of mating experience, since males who have mated but are not exposed to females afterward still fight over females, while males who do not mate but are exposed to females show suppression of fighting (Yuan et al., 2014). Interestingly, the males who are exposed to females for 24 hours still show a high level of courtship toward them, suggesting that the effect of inhibition of aggression is not due to desensitization toward females (Yuan et al., 2014). It is worth noting that *ppk29* mutant flies show reduced courtship toward females (Thistle et al., 2012); thus, it is unclear why *ppk29* mutant flies and flies whose *ppk29*+ neurons are silenced still fight over females (Yuan et al., 2014). Males detect the presence of females via the detection of female-specific cuticular hydrocarbons—7,11-heptacosadiene (7,11-HD) and 7,11-nonacosadiene (7,11-ND)—known to produce courtship behaviors in male flies (Antony and Jallon, 1982; Ferveur, 2005; Jallon, 1984). 7,11-HD and 7,11-ND are detected by *fruitless*+, *ppk23*+, *ppk29*+, *ppk25*+, *DEG/ENaC*+, *CheB42*+, *nope*+ GRNs in the leg, as well as some GRNs in the labellum (Lin et al., 2005; Lu et al., 2012; Pikielny, 2010; 2012; Starostina et al., 2012; 2009; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014; Yuan et al., 2014). These data suggested the possibility that flies may use 7,11-HD in order to detect

females as a possible resource to fight over, and that they may accomplish this by using *ppk23*+ GRNs.

In addition to females, *Drosophila melanogaster* males also fight over food (Chen et al., 2002; Hoffmann and Cacoyianni, 1990; Skrzipek et al., 1979). In particular, Hoffmann and Cocoyianni as well as Skrzipek and Kröner found that a group of male flies in the presence of food *and females* fight over food in a food size-dependent manner (Hoffmann and Cacoyianni, 1990; Skrzipek et al., 1979). Fly food or other edible substance that flies fight over, such as apple, banana, orange, melon, and lemon (Hoffmann and Cacoyianni, 1990), are chemically complex. Thus far specific chemicals, which increase aggression in *Drosophila,* are not known. Food activates multiple sensory systems, including the gustatory system (Meunier et al., 2000; Thorne et al., 2004; Ueno et al., 2001; Wang et al., 2004) as well as the olfactory system (Hallem and Carlson, 2006; Wang et al., 2003). Sweet-tasting compounds are detected by *Gr5a*+, *Gr64a-e*+ GRNs (Dahanukar et al., 2001; 2007), while volatile compounds present in the food activates many populations of ORNs (Hallem and Carlson, 2006; Semmelhack and Wang, 2009; Wang et al., 2003) (reviewed in [Vosshall and Stocker, 2007]). The behavioral role of these food-activated sensory neurons have been studied in the context of feeding-related behaviors, such as proboscis extension reflex (PER) (Dethier, 1976) or food preference assays (Wang et al., 2004), but their role in detecting food compounds suggested the possibility that they may play a role in food-promoted aggression.

General issues with studying resource-mediated aggression and gaps in knowledge

There are several general issues that are potential confounds or critical missing information in studying the effects of specific resources on aggression, detailed further below: 1) Problem of correlation. 2) Problem of attraction. 3) Problem of specificity. 4) Missing key information: specific sensory cues and sensory neurons. 5) Missing key information: dose-response curve and quantitative explanations using game theory. 6) Misuse of the word territoriality.

1) Problem of correlation: Many studies report correlations between the presence of resource and aggression from observational studies. However, these studies often lack experimental manipulations of the resources themselves, which are necessary to prove that they are necessary and sufficient. In particular, studies in *Drosophila melanogaster* (Chen et al., 2002; Hoffmann and Cacoyianni, 1990; Yuan et al., 2014) use both females and food to measure aggression, which leaves the possibility that food or females individually may not increase aggression.

2) Problem of attraction: Since resources such as food and females are attractive in all or nearly all species, the effects seen on aggression may be indirect due to an increase in encounter rates between contestants. At minimum, encounters between contestants must be quantified with aggression simultaneously to test whether food or females increases rate, frequency and duration of encounters. In

addition, when appropriate, aggression should be normalized by encounter duration so that the effects of increasing chance interaction between males is not the principal cause of aggression.

3) Problem of specificity: Food and females have been shown to increase many behaviors, including locomotion and mating; thus, it is unclear food increases general arousal, or social arousal or aggression-specific arousal. For instance, crayfish fight more in the presence of food (Stocker and Huber, 2001), but because they also move more, the authors concluded in this case that the increased fighting may be due to increased encounters. In almost all other studies, no information is given regarding the effects of a resource on non-aggressive behaviors. In *Drosophila melanogaster*, food is known to increase locomotion (unpublished data) as well as male-female courtship (Grosjean et al., 2011), suggesting the possibility that food may increase all behaviors, and not just aggression. If food simply increases all behaviors, this would lead to the conclusion that food does not increase competition *per se*, but rather it increases arousal or social arousal. At minimum, other behaviors should be quantified in parallel with aggression and when appropriate, aggression should be normalized by locomotion as it has been done before in *Drosophila* (Hoyer et al., 2008).

4) Missing key information–specific sensory cues and sensory neurons: No study to date studying the effects of resource on aggression narrows down the effect to a single molecule and neural circuits processing these cues. In rare cases, where molecular identity of an aggression-promoting sensory cue was identified, such as

β-MSP in squids, the neural circuits processing these cues remain unknown, as not all organisms are amenable to molecular dissection of neural circuitry (Cummins et al., 2011). Furthermore, to date, there is no study identifying specific sensory neurons, which detect resource cues and are involved in resource-promoted aggression. Instead, sensory neurons detecting food and female cues are studied only in the context of feeding and courtship, leaving open the possibility that different sensory neurons may play a role in aggression. This is critical in the context of studying how neural circuits control decision-making, since identification of specific resource cues and receptor neurons detecting these cues is a necessary first step to understanding how the central processing in the brain uses this sensory information to produce the behavioral decision to fight.

5) Missing key information—dose-response curve and quantitative models: The dose-dependent relationship between resource abundance and aggression is either not known or only partially known in most cases. Understanding this relationship is critical to characterizing the input-output relationship between resource and behavior. Quantitative characterization of resource inputs for aggression can be used to test whether animals use strategic decision-making, as predicted by game theoretic models. Game theoretic models predict that animals make cost-benefit calculations, and characterizing these parameters in aggression should set some constraints for how the brain may make such cost-benefit calculations.

6) Misuse of the word territoriality: Territoriality is often a word that is used

synonymously to aggression. Nevertheless, not all species that exhibit interspecific aggressive behaviors are territorial. Some social species, such as humans and chimpanzees, live together in groups occupying large territories and exhibit inter-group aggression (Wrangham et al., 2006). Other species such as birds, mice and shellfish defend the physical spaces where they reside. In such species, aggression could be interpreted as *territorial*. In *Drosophila,* although the term territoriality is frequently used when referring to aggression (Chen et al., 2002; Hoffmann and Cacoyianni, 1990), previous studies have not distinguished between the defense of a territory (territoriality) from the defense of a resource per se (Dow and Schilcher, 1975; Jacobs, 1960). To demonstrate *bona fide* territoriality, it would be necessary to show that animals defend a physical space (territory) or a border surrounding such a space, rather than just the resource.

My thesis attempts to answer all these questions in the organism *Drosophila melanogaster.*

Chapter 2

HOW FOOD CONTROLS AGGRESSION IN *DROSOPHILA MELANOGASTER*

Title Page

Title: How food controls aggression in *Drosophila*

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Abstract

How animals use sensory information to weigh the risks vs. benefits of behavioral decisions remains poorly understood. Inter-male aggression is triggered when animals perceive both the presence of an appetitive resource, such as food or females, and of competing conspecific males. How such signals are detected and integrated to control the decision to fight is not clear. For instance, it is unclear whether food increases aggression directly, or as a secondary consequence of increased social interactions caused by attraction to food. Here we use the vinegar fly, *Drosophila melanogaster,* to investigate the manner by which food influences aggression. We show that food promotes aggression in flies, and that it does so independently of any effect on frequency of contact between males, increase in locomotor activity or general enhancement of social interactions. Importantly, the level of aggression depends on the absolute amount of food, rather than on its surface area or concentration. When food resources exceed a certain level, aggression is diminished, suggestive of reduced competition. Finally, we show that detection of sugar via Gr5a^{+} gustatory receptor neurons (GRNs) is necessary for food-promoted aggression. These data demonstrate that food exerts a specific effect to promote aggression in male flies, and that this effect is mediated, at least in part, by sweet-sensing GRNs.

Introduction

Metazoan organisms in nature constantly face behavioral choices. Depending on the actions selected, an animal may gain access to potential resources or risk starvation, predation or agonistic interactions. Aggression is an ideal system in which to study how the nervous system makes value-based decisions, as the decision to fight comes with apparent costs and benefits, and requires the assessment of a potential conflict: the detection of attractive resources and competitors who limit access to such resources.

As in many other species, *Drosophila* males exhibit a gender-specific repertoire of stereotyped aggressive behaviors (Asahina et al., 2014; Chen et al., 2002; Fernández et al., 2010; Jacobs, 1960; Kurtovic et al., 2007; Nilsen et al., 2004; Vrontou et al., 2006; Wang and Anderson, 2010; Wang et al., 2011). Recent studies have identified some of the male-specific sensory signals and their physiological receivers relevant for aggression (Billeter and Levine, 2012; Billeter et al., 2009; Fan et al., 2013; Fernández et al., 2010; Fernández and Kravitz, 2013; Lacaille et al., 2007; Lu et al., 2012; Pikielny, 2012; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014; Wang and Anderson, 2010; Wang et al., 2011). In particular, cuticular hydrocarbon pheromones, such as 11-*cis*-vaccenyl acetate (cVA) (Asahina et al., 2014; Chen et al., 2002; Chyb et al., 2003; Dahanukar et al., 2001; Hoffmann and Cacoyianni, 1990; Hoyer et al., 2008; Skrzipek et al., 1979; Wang et al., 2011; 2004; Yuan et al., 2014) and (*z*)-7-tricosene (7-T) (Dahanukar et al., 2007; Dethier, 1976; Fernández et al., 2010;

Inagaki et al., 2012; Slone et al., 2007; Svetec et al., 2005; Wang et al., 2011; 2004) promote aggression through olfactory (Billeter and Levine, 2012; Billeter et al., 2009; Fan et al., 2013; Fernández et al., 2010; Fernández and Kravitz, 2013; Kurtovic et al., 2007; Lacaille et al., 2007; Lu et al., 2012; Pikielny, 2012; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014; Wang and Anderson, 2010; Wang et al., 2011) and gustatory receptor neurons (Billeter and Levine, 2012; Chyb et al., 2003; Fan et al., 2013; Fernández and Kravitz, 2013; Lu et al., 2012; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014). However, the detection of cues from conspecific males is a necessary but not sufficient condition for aggression: male flies will not fight unless a resource, such as food or females, is present (Asahina et al., 2014; Chen et al., 2002; Dahanukar et al., 2007; Dethier, 1976; Fernández et al., 2010; Hoffmann and Cacoyianni, 1990; Hoyer et al., 2008; Inagaki et al., 2012; Skrzipek et al., 1979; Slone et al., 2007; Svetec et al., 2005; Wang et al., 2011; 2004; Yuan et al., 2014).

Despite much progress, fundamental questions remain unanswered about how resources promote aggression. In particular, it is widely assumed that flies fight in the presence of food due to competition over a limiting resource or to claim territory for potential reproductive advantages (Chen et al., 2002; Dow and Schilcher, 1975; Hoffmann and Cacoyianni, 1990; Sweeney et al., 1995; 2011). However, other explanations have not been excluded. For example, increased aggression in the presence of food could simply be due to an increase in

encounter frequency and/or duration between males attracted to the resource, or to an increase in aggressive drive or arousal. Food may also increase locomotor activity, promoting increased encounters and thereby indirectly enhancing aggression. In addition, most previous reports (Chen et al., 2002; Dahanukar et al., 2007; Dethier, 1976; Gordon and Scott, 2009; Hoffmann and Cacoyianni, 1990; Inagaki et al., 2012; 2013; Kang et al., 2011; Keene and Masek, 2012; Lu et al., 2012; Marella et al., 2006; Skrzipek et al., 1979; Slone et al., 2007; Wang et al., 2004) measured male-male aggression in the presence of females, which added a potential confound, as presence of females can increase aggression on its own (Harris, 2010; Yuan et al., 2014; Zhou et al., 1997). Finally, it is not clear whether food promotes aggression in a purely permissive or in an instructive manner.

A resolution of these issues would be facilitated by a quantitative analysis of aggressive behavior on variable food resources. Such analyses have been enabled by the development of machine vision-based automated aggressive behavior recognition software (Dankert et al., 2009; Gray et al., 2002; Hoyer et al., 2008; Manzo; Zhang et al., 2013). Here we report on the results of such an analysis, performed in the context of systematic and quantitative manipulations of food resource parameters and analyses of their effects on male-male social interactions. Our results set constraints, in a principled and rigorous manner, on models for how food promotes aggression. We also identify a key component of food and its chemoreceptor that are required for aggression.

Results

The effect of food to promote aggression is not due to an increase in male-male social encounters

Previous reports (Armstrong, 1991; Chen et al., 2002; Dow and Schilcher, 1975; Hoffmann and Cacoyianni, 1990; Skrzipek et al., 1979) on food's influence on fly aggression used assays with females, leaving open the possibility that food only exerts influence on aggression in the presence of females. Recently, a paper in our laboratory (Asahina et al., 2014; Santangelo et al., 2002) showed that in a small arena without females, food increases aggression in a pair of males. We investigated whether the presence of a central food patch in a bigger arena (as described in [Cummins et al., 2011; Hoyer et al., 2008]) could increase aggression compared to agarose, and observed an increase in the number of lunges in the presence of food (Figure 1a, apple juice mixed with 100 mM sucrose and 1% agarose is hereafter referred to as "food"; different from fly culture medium).

Fly aggression assays are typically performed in the presence of a small central food patch (Hoyer et al., 2008; Rypstra et al., 2009) or an elevated cup containing food (Chen et al., 2002; Hoffmann and Cacoyianni, 1990; Holldobler and Lumsden, 1980; Mundiyanapurath et al., 2007; Skrzipek et al., 1979), placed in a larger chamber (Figure 1c, left and Figure S4a). Since food is an attractive resource (Guerra and Mason, 2005; Root et al., 2011), it is possible that food increases aggression by simply increasing the proximity between the two flies due

to their attraction to food. This increase in proximity could in turn increase the frequency or duration of encounters between flies. As aggressive interactions between males depend on non-volatile cuticular hydrocarbon pheromones that are detected by contact chemoreceptors (Chen et al., 2002; Fan et al., 2013; Fernández et al., 2010; Hoffmann and Cacoyianni, 1990; Lu et al., 2012; Skrzipek et al., 1979; Thistle et al., 2012; Toda and Zhao, 2012; Toda et al., 2012; Ueda and Kidokoro, 2002; Wang et al., 2011; Watanabe et al., 2011), an increase in encounters might enhance aggression indirectly, by promoting pheromone detection. In order to distinguish whether the effect of food to enhance aggression was due to an increased fly proximity on the food patch, we repeated the assays in a modified arena in which the entire surface was covered with a food substrate (Figure 1c and Figure S4b). Control arenas were covered with a uniform layer of agarose. Under these conditions, there was still a clear and significant effect of food to increase the number of lunges (Figure 1b).

To gain further insight into how food affects the proximity of flies and how this may affect the level of aggression, we examined a heat map of fly distribution in the presence of a patch of food and uniform food (Figure 1c). As expected, a central food patch in aggression assays increased the density of flies in this local area (Figure 1c left and Figure S1a), but in an arena containing uniform food, flies were not localized in any particular spot (Figure 1c right and Figure S1b).

To quantify the effects of aggregation on proximity between two flies, we measured the amount of time flies spent at various distances from each other (Figure 1d). This histogram revealed a prominent peak at an inter-fly distance of 3-

5mm, suggesting that flies have a preference to remain within 1-2 body lengths (depending on orientation, average male fly body length is ~2.5mm). The height of peak was the same whether uniform food was present or absent (Figure 1d). In contrast, in the presence of a small food patch, there was a small but statistically significant increase in the height of the interaction peak (Figure S1e). This peak likely reflects a preferred interaction distance, as transformation of one fly's position with respect to time by reversing the order (first frame becomes the last frame of the assay) or shifting the order (first frame becomes the 1000th frame) while keeping the other fly's position constant led to a completely different inter-fly distance distribution (Figure S1f and Figure S1g). In order to convert this distribution to a single metric, we integrated the area under the peak between 0 to 10mm (3-4 body lengths depending on the orientation of the two flies), which we operationally define as "encounter duration," which accounts for roughly 50% of the time flies spend during the assay. This parameter was not significantly different between uniform food vs. agarose (Figure 1e), further confirming that food is able to increase aggression without affecting proximity and encounter parameters. Encounter duration was a more robust measure of proximity than other measurements of proximity, such as encounter frequency, because encounter duration displayed less variance, was uncorrelated with aggression (Figure S2a) and contained temporal information (i.e. long encounter vs. a short encounter). Taken together, these data indicate that the presence of food can increase aggression independently of any effect to increase the average time that flies spend in proximity to each other.

Food increases male-male aggression independently of arousal

The foregoing analysis left open the possibility that food might promote aggression by increasing general arousal. One measure of general arousal is locomotor activity (Nitz et al., 2002; van Swinderen and Andretic, 2003). Indeed, a pair of male flies exhibited a small but significant increase in distance traveled in the presence vs. the absence of food (Figure 1f). Because aggression itself involves increased locomotion (Figure S3a) (Dankert et al., 2009; Hoyer et al., 2008), it is not clear whether increased locomotion is a cause or a consequence of increased aggression. Previous studies have addressed this by normalizing the number of lunges to total distance traveled (Dankert et al., 2009; Hoyer et al., 2008). Normalized for locomotion, food still robustly increased aggression (Figure 1g).

If food increases aggression by increasing general or social arousal, it might also be expected to increase male-male courtship, another social behavior observed in these assays (Billeter et al., 2009; Certel et al., 2007; Dankert et al., 2009; Fernández et al., 2010; Fernández and Kravitz, 2013; Svetec et al., 2005; Thistle et al., 2012; Wang and Anderson, 2010; Wang et al., 2011; 2008). Male-male courtship is known to be inhibited by male-specific pheromones (Antony and Jallon, 1982; Billeter et al., 2009; Wang et al., 2011), but it is still observed among pairs of wild-type male flies albeit at low frequency (Certel et al., 2007; Cobb and Jallon, 1990; Dankert et al., 2009). Unlike male-male aggression, food did not increase male-male courtship, measured by unilateral wing-extensions (Figure 1h)

and circling behavior after normalization for distance traveled (Figure 1i).

Male-male courtship occurs predominantly in the first few minutes of a social encounter, and therefore, averaging over the entire 20-minute assay might have missed a transient food-dependent increase (Figure S3b). As expected, food increased aggression in the first three minutes (Figure S3c). In contrast, food actually decreased the frequency of one-wing extensions over the first three minutes of the assay (Figure 1j and Figure S3d). Thus, in pairwise male-male social encounters, food selectively enhances aggression, but not male-male courtship. These results support the notion that food can specifically increase aggression in a manner that does not reflect a general increase in social interactions.

The level of aggression depends on the absolute amount of food

If food specifically enhances aggression, how do flies measure it? The answer to this question sets constraints on the sensory systems that are involved, and ultimately how the brain uses this information to guide the decision to fight. We first examined the effect of changing the area over which food (at a fixed concentration) is distributed, using a modifiable arena (Figure S4c). Consistent with previous reports (Hoffmann and Cacoyianni, 1990; Skrzipek et al., 1979), we observed a dose-dependent relationship between the size of the food patch and the level of aggression (Figure 2a). Next, we investigated whether this dose-dependent increase was due to an effect on proximity, arousal, or general social interactions. Although, we observed a slight increase in locomotion as the size of

the food patch increased (Figure 2b), this enhanced aggression was seen even when normalized by locomotion (Figure 2c). Furthermore, the inter-fly distance distribution was not changed by any of the differently sized food arenas that were tested (Figure S5b). Unlike aggression, male-male courtship showed no change in response to the change in the amount of food (Figure 2d), suggesting that the dose-dependent effect of food does not reflect a general increase in social interactions.

Previous studies did not distinguish whether the increase in aggression caused by increasing the size of food patch was due to an increase in area, total food amount or both (Hoffmann and Cacoyianni, 1990; Skrzipek et al., 1979). We therefore investigated whether changing the concentration of food while keeping the arena area constant would yield a similar result. Indeed, aggression in a fixed-size arena increased as the concentration of food increased (Figure 2e left). In fact, when we compared the level of aggression in the cases where the areas of food were different (Figure 2e right) but the caloric content was matched, the level of aggression was indistinguishable (see Figure S5a for side-by-side comparisons). These data are incompatible with the notion that flies assess the quality of food in the context of aggression by using a physical dimension of food territory, such as area or perimeter circumference. Instead, these results suggest that the level of aggression depends upon the absolute amount of food in the substrate.

Flies decrease fighting when food exceeds a certain threshold

The foregoing experiments show that aggression requires a minimal amount of food, and scales as the quantity of food increases. If aggression is driven by competition over food, then aggression should decrease at some point if the food becomes available in excess, as it is seen in many other species (Hixon et al., 1983; Smith and Price, 1973). Indeed, previous studies showed that a very large area of food can decrease aggression in comparison to an intermediate area of food (Hoffmann and Cacoyianni, 1990). We confirmed these findings in our setup by testing 5 additional larger food patches with areas > 707 mm^2. Under these conditions, we observed a gradual decrease in aggression as the area of the food patch was increased to 2376 mm^2, the largest size tested (Figure 3a and Figure SS4c).

It was previously suggested that the decrease in aggression observed may be due to the increased energetic cost of defending a greater territory or a larger food patch (Hoffmann and Cacoyianni, 1990). However, given our finding that fly aggression depends on the absolute amount of food rather than the area of food, it remained a possibility that the decrease in aggression was also caused by a greater quantity of food. Indeed, when we decreased the concentration of food in the largest arena (2376 mm^2 arena) from 100% to 30%, aggression was increased to a level equivalent to that in a smaller (707 mm^2) but nutritionally identical arena containing 100% food (Figure 3d). This increase in aggression was still significant after normalization for locomotion (Figure 3e and Figure S5c), while male-male courtship did not show any increase (Figure 3f). These data further support the idea that flies tune their level of aggression as a function of the absolute amount of

food available. Aggression is enhanced as the amount of food is increased to a certain point, and decreases as the amount of food is increased above that amount.

The dose-response relationship we observed above suggested that there could be a continuous relationship between the amount of food and aggression. This would imply that the role of food may be instructive rather than purely permissive. Nevertheless, using the Kruskal-Wallis test, we were only able to resolve a few statistically distinct groups among the different sizes of food tested, due to the high pair-to-pair variability in the amount of fighting (Table S3). One shortcoming of using Kruskal-Wallis test is that since it treats groups being tested as categorically distinct, as the number of groups increases, Bonferroni corrections for multiple comparisons reduce statistical power to resolve small differences. For instance, among the 13 different sizes of food we tested, there were 78 comparisons made, and after correcting for multiple comparisons, only a few points were statistically significantly different from each other, despite the fact that when individually tested in a pair-wise manner, many more were significantly different (See Table S3).

As an alternative approach to this problem, since the amount of food is a continuous rather than a discrete variable, we performed a curve-fitting analysis to model the relationship between food quantity and aggression. The simplest possible model to test whether the data we observe has an increasing phase and a decreasing phase is the quadratic function (Figure S6a). We ran an ordinary least squares estimation method, a form of regression analysis, among quadratic

functions, to find the coefficients β_0, β_1, and β_2, which best fit the data. The results (Figure S6b) suggested that 1) There is a non-random relationship between the amount of food and aggression, and 2) there is an inverse-U shaped relationship between the amount of food and aggression. That is, since the coefficient β_0 is significantly different from 0, it implies that the as food increases, aggression goes up until it reaches a certain threshold, and then goes down. The 99% confidence intervals for the coefficients β_0, β_1, and β_2 show that the model predicts an *X*-intercept of 14 to 26 (14 to 26 lunges when there is no food) and an inverse-U shape (99% confidence interval for β_0 is bound within negative values). The results of the analysis were statistically significant for the joint F-test for coefficients β_0, β_1, and β_2, which suggests that there is a non-random relationship between aggression and the amount of food. Since the coefficient β_0 is significantly different from 0, a quadratic function yielded a higher fit to the data than a linear function (Figure S6c). This analysis suggests that aggression exhibits a continuous increase and then a decrease as the quantity of food is increased, rather than having an all-or-none effect.

While aggression showed an inverse U-shaped curve in response to increasing amount of food (Figure 3a, 3b, Figure S6b and Table S3), locomotion (Figure S5c and Table S2) and male-male courtship (Figure S5d and Table S4) showed no such patterns, suggesting that the biphasic response is specific to aggression. Encounter duration was slightly different when compared to the no-food conditions (Figure S5d), although the overall inter-fly distance distribution remained unchanged (Figure S5b). These data confirm and extend the results of

the previous finding (Hoffmann and Cacoyianni, 1990), but are inconsistent with their interpretation that a larger size of food decreases aggression due to the increased energetic cost of defending a larger territory. Instead, we favor the idea that aggression between flies reflects competition over limiting amounts of food resources, which can be partially overcome when nutrients exceeds a certain threshold.

Flies display territorial behavior

Territorial behavior refers to overt or implied defense of an area by one or a group of animals at the exclusion of others (Adams, 2001). Although the term territoriality is frequently used when referring to aggression in *Drosophila* (Chen et al., 2002; Hoffmann and Cacoyianni, 1990), previous studies have not distinguished between the defense of a territory (territoriality) from the defense of a resource per se (Dow and Schilcher, 1975; Jacobs, 1960). To investigate this issue, we observed in more detail the spatial distribution of a pair of flies with respect to food resources of different areas.

As mentioned earlier, flies preferentially occupy the area where food is present (Figure 1b and 4a). In addition, we observed that as the area of the food patch was increased, the position heat map showed an apparent circular “donut” shape (Figure 4a), suggesting an increased preference of flies to remain near the periphery of the food patch. This observation suggested that flies may defend the perimeter of the food, rather than the entire food resource, when the size of the patch is large.

To distinguish whether this phenomenon was related to aggression, or simply reflected an innate preference of flies to occupy the boundary of a food patch, we compared the distribution of single flies and fly pairs for two different sizes of food patches (Figure 4b). In order to quantify these distributions with respect to the food patch area, we measured the amount of time flies spent as a function of the distance from the food patch border patches, and aligned the histograms to the border defined as 0mm (Figures 4c and 4d). In both 30mm and 45mm diameter patches, we observed two peaks defining three zones in the histograms, which we refer to as Zones A, B, and C (Figures 4c and d, lower). Zone A comprised the food patch itself, and exhibited a peak in the fly distribution at the border. Zone B comprised the area between the food border peak and a second peak, located approximately 15-20 mm from the outside edge of the arena. Zone C comprised the perimeter area of the arena. Since Zone A was the area occupied by the food patch, fly occupation of this area simply reflected their natural attraction to food. Zone C could, in part, reflect thigmotactic tendencies of flies (Martin, 2004; Simon et al., 2010), since in the absence of food, a similar peak around 15-20 mm from the edge of the arena was also observed (Figure S7a). To investigate whether these experimental peaks were different from a random distribution, which would be expected if flies behaved as if they were randomly moving particles, we calculated a random distribution from the area in the bins at each indicated distance from the food border and compared it to the experimental distribution (Figure S7b). These comparisons revealed that in the absence of a food patch (blue line), flies behaved similarly to randomly moving particles (teal-

colored line). In contrast, in the presence of a 30mm-diameter food patch, fly positions (orange) were not randomly distributed.

In both single and paired fly experiments, there were two peaks dividing these three zones in both 30mm-diameter (Figure 4c, blue for single fly and orange for paired fly experiments) and 45mm-diameter food patches (Figure 4d). Nevertheless, we observed a noticeable difference in the distribution of flies within Zone B. Pairs of flies appeared to spend more time in this zone than did single flies. To quantify these differences, we calculated the area under the curves in Zone A and Zone B for single vs. paired flies. Single male flies spent significantly less time than did flies in pairs in Zone B for both 707mm^2 and 1590mm^2 food patches (Figure 4e). In contrast, when we calculated the amount of time flies spent in the food area (Zone A), we found that the presence of an opponent male made no difference (Figure 4f). These data indicate that the presence of an opponent does not enhance attraction to food; instead, it only increases the amount of time flies spend in the area just outside the food border, suggesting that fighting flies adopt a “perimeter defense” strategy. These data are consistent with the notion that when the size of the food patch is large (Figure 4a, 177 mm^2 vs. 1590mm^2), *Drosophila* males fight over access to a food-containing territory, rather than just over the food resource itself.

Sucrose is sufficient to promote aggression

Foregoing data suggested that flies may use their chemosensory systems to measure the absolute nutritional content of the food to tune the level of aggression.

Apple juice and fly culture food are complex mixtures containing a variety of odorants and tastants (Leopold et al., 2011; Lewis, 1960). One obvious indicator of nutritional content in natural food resources is the concentration of sugar. Therefore, we tested whether pure sucrose, present in fly culture medium and food mix used in our experiments, would be sufficient to increase aggression in the absence of any other food component. Surprisingly, we found that a small patch of 100mM sucrose (see Figure S4e), comparable to concentrations found in fruits (USDA, 2011) and in laboratory fly food medium (Lewis, 1960), was sufficient to promote aggression to a level comparable to that observed using the food substrate (Figure 5a and Figure S8d). Similar to uniform food, the ability of sucrose to increase aggression was not due to a difference in the encounter duration, because the presence of a patch of sucrose neither changed the overall distribution of the flies (Figure 5b), nor changed the encounter duration (Figure 5c and 5d). The presence of sucrose increased locomotion (Figure 5e), but the increase in aggression caused by sucrose remained significant following normalization to distance traveled (Figure 5f). In contrast, male-male courtship was not increased (Figure 5g). Thus, pure sucrose can mimic the effect of food to increase aggression.

To examine the dose-dependency of aggression on sucrose, we compared the number of lunges in 100, 200 and 800 mM sucrose (Figure 5h, see Figure S4e). Similar to the results obtained with food (Figure S8d), we first saw an increase in aggression when we increased the concentration of sucrose from 100 to 200 mM. Moreover, when we further increased the level of sucrose to 800 mM,

the level of aggression was no different from the control condition (Figure 5h). Taken together, these data suggest that sucrose exhibits a bi-modal influence on aggression that is qualitatively similar to that seen with food.

The activity of sugar sensing *Gr5a*$^{+}$ gustatory receptor neurons is required for aggression

Previous work has shown that several subpopulations of fly gustatory receptor neurons play a role in male-male aggression and male-male courtship via detection of pheromones (Billeter and Levine, 2012; Fan et al., 2013; Fernández et al., 2010; Fernández and Kravitz, 2013; Lu et al., 2012; Pikielny, 2012; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014; Wang et al., 2011). Sucrose is known to be detected by *Gr5a*$^{+}$ GRNs in the fly gustatory system (Chyb et al., 2003; Dahanukar et al., 2001; Wang et al., 2004). However, these GRNs have previously only been implicated in the context of feeding and proboscis extension behaviors (Dahanukar et al., 2007; Dethier, 1976; Inagaki et al., 2012; Slone et al., 2007; Wang et al., 2004). Because we found that sucrose is sufficient to promote male-male aggression, we investigated whether the activity of *Gr5a*$^{+}$ GRNs is required for male-male aggression on food. To test this, we silenced the neurons by expressing tetanus toxin light chain (TNT) (Sweeney et al., 1995) under the control of the Gr5a-GAL4 promoter [31].

First, we verified that silencing the *Gr5a*$^{+}$ GRNs via expression of TNT reduced sucrose sensitivity by performing proboscis extension reflex (PER) assay (Figure 5i), as described previously (Dahanukar et al., 2007; Dethier, 1976;

Inagaki et al., 2012; Slone et al., 2007; Wang et al., 2004). Next we tested the effect of silencing *Gr5a*$^{+}$ GRNs on aggression and found that the activity of *Gr5a*$^{+}$ GRNs is necessary for aggression on food (Figure 5j). Importantly, flies whose *Gr5a*$^{+}$ GRNs were silenced could still perform aggression at a level comparable to the genetic controls in the presence of females, suggesting that the effect of silencing *Gr5a*$^{+}$ GRNs did not merely impair the ability to fight (Figure S8a**)**. We confirmed that we could get the same result of reduced aggression in the presence of food using another effector, UAS-Hid (Zhou et al., 1997), which was shown to disrupt the function of *Gr5a*+ GRNs (Manzo; Zhang et al., 2013) (Figure S8b). Since food contains various gustatory and olfactory cues (2011) that are not detected by *Gr5a*$^{+}$ GRNs, these data suggest that detection of sweet tastants plays a permissive role in food-induced aggression.

Finally, we investigated whether increasing the activity of *Gr5a*$^{+}$ GRNs would suffice to increase aggression. To do this, we expressed different effectors, that increase the neuronal activity in *Gr5a*$^{+}$ GRNs, including UAS-DTRPA1, UAS-TRPV1, UAS-NaChBac tub-Gal80ts, UAS-ChR2 and UAS-ReACh (Gordon and Scott, 2009; Inagaki et al., 2012; 2013; Kang et al., 2011; Keene and Masek, 2012; Lu et al., 2012; Marella et al., 2006). However, none of the effectors increased aggression in the absence of food (Figure S8c; UAS-dTrpA1, UAS-ChR2 and UAS-ReACh data not shown**)**. This was true even for TRPV1, a cation channel activated by the ligand capsaicin, which was added to the agarose substrate in order to ensure activation of *Gr5a*$^{+}$ GRNs on the tarsae (Figure S8c). These data suggest that although *Gr5a*$^{+}$ GRNs are necessary for normal levels of

food-induced aggression, they are not sufficient to increase aggression in the absence of food.

Discussion

In nature, when confronted with another animal, a male has to decide whether to engage in social behavior and if so, whether to engage in aggression or courtship. Understanding how information processing in the brain controls such behavioral decisions is a fundamental problem in neurobiology. An essential first step in this framework is to identify the relevant sensory cues to a particular behavior and neural circuits, which process these inputs.

Intraspecific aggression is an innate social behavior observed in many species. The presence of either food or a mating resource is fundamental to releasing aggression, as a link between these resources and aggression has been observed in many species, such as primates (Harris, 2010), mice (Gray et al., 2002), birds (Armstrong, 1991), fish (Santangelo et al., 2002), squid (Cummins et al., 2011), spiders (Rypstra et al., 2009), ants (Holldobler and Lumsden, 1980), cockroaches (Guerra and Mason, 2005), and flies (Chen et al., 2002; Hoffmann and Cacoyianni, 1990; Skrzipek et al., 1979; Ueda and Kidokoro, 2002).

In flies, correlations have been observed between an increased probability of aggressive encounters and the presence of females or various food substrates (Billeter et al., 2009; Chen et al., 2002; Fernández et al., 2010; Fernández and Kravitz, 2013; Hoffmann and Cacoyianni, 1990; Lacaille et al., 2007; Skrzipek et

al., 1979; Wang and Anderson, 2010; Wang et al., 2011; Yuan et al., 2014). Nevertheless, as most studies investigated aggression in the presence of both food and a female, until recently (Asahina et al., 2014; Wang et al., 2011), no study has compared the level of aggression with food vs. no food in the absence of females. Furthermore, no study has distinguished whether attractive resources directly promote male-male aggression in *Drosophila,* or rather promote this behavior indirectly simply by increasing the proximity and therefore the probability of encounter between competing males (Chen et al., 2002; Fernández et al., 2010; Hoffmann, 1987; Hoffmann and Cacoyianni, 1990; Jacobs, 1960; Svetec et al., 2005; Wang et al., 2011). In addition, it was not clear whether food increased all social behaviors, or specifically increased aggression. Resolving these issues is fundamental to studies of aggression in all animals.

Here we confirm that in flies, food can increase aggression relative to an agarose substrate in the absence of females (Asahina et al., 2014; Kurtovic et al., 2007; Wang and Anderson, 2010). Furthermore, we provide evidence that this effect is not due to an increase in the proximity of flies to each other: food covering the entire surface of the arena does not increase encounter duration, but nevertheless increases aggression. Our data also indicate that although food slightly increases locomotor activity, its effect to increase aggression is still significant even after normalizing for locomotion. In contrast to aggression, male-male courtship is unchanged or even somewhat decreased by the presence of food. Taken together, these data suggest that food specifically promotes

aggression.

Previous studies have demonstrated that there is a food-patch-size-dependent increase in aggression in flies (Hoffmann and Cacoyianni, 1990; Skrzipek et al., 1979). However, it was unclear from these studies whether the flies were responding to an increase in the amount of food, or rather the area of food. We systematically compared increases in both food area at a fixed concentration, and increased food concentration in an arena of fixed area. Both manipulations increased the amount of aggression (up to a certain point), indicating that the relevant factor is the absolute amount of food, rather than the area over which it is distributed. Importantly, above a certain amount of food, aggression is decreased, while decreasing the concentration of food in the same-size arena increased aggression. These data reveal a dose-response relationship between food and aggression, suggestive of competition.

Since we observed multiple incremental steps in the level of aggression as the amount of food was increased, food seems to play an instructive role in promoting aggression rather than a purely permissive role. In the latter case, there would be only two statistically-distinguishable levels of aggression: high when there is any amount of food, and low when there is no food. Nevertheless, because there is a large amount of pair-to-pair variation in aggression, the change in aggression can only be detected between large changes in the amount of food. It is unclear why a male fly, whose length is approximately 2.5mm, continues to increase aggression until the diameter of the food patch reaches 30mm, and only

decreases aggression slightly when the diameter of food exceeds 50mm (a circular patch 25x length of the fly body). The exact mechanism by which flies "measure" the absolute amount of food to tune the level of aggression is unclear.

We also found that sucrose, which is present in many fruits, fly medium, and the food in our assay, mimic food's effects on aggression (Lewis, 1960). There is a dose-dependent increase in aggression and eventual decrease after the amount of sucrose exceeds a certain amount, similar to the effect of food. By inhibiting *Gr5a*$^{+}$ GRNs, the sweet-sensing gustatory receptor neurons in flies, we showed that the sugar-sensing gustatory receptor neurons play a permissive role in aggression promoted by food. Artificial activation of *Gr5a*$^{+}$ GRNs failed to increase aggression, however. This result, taken at face value, would seem to suggest a permissive and not instructive role for sugar in aggression, in seeming contradiction to the result of our dose-response studies. The reasons for our failure to show that artificial activation of *Gr5a*$^{+}$ GRNs is sufficient to increase aggression may be technical or biological. Technical reasons could include an inability to activate *Gr5a*$^{+}$ GRNs to a critical threshold necessary for aggression, perhaps due to a depolarization block (Inagaki et al., 2013). Alternatively, *Gr5a*$^{+}$ GRNs may be required to detect the presence of sugar, but the calculation of relative resource value may require higher order circuits. It is worth noting that sucrose is attractive to egg-laying females (Schwartz et al., 2012), much like various types of fruits (Hoffmann and Cacoyianni, 1990). This suggests the possibility that male flies may compete over food not only to gain access to nutrients, but also to locations where egg-laying females are present. Consistent with this idea, food also increases male-female

courtship (Grosjean et al., 2011).

A potential caveat regarding our experiments with *Gr5a*$^{+}$ GRNs is that our GAL4 driver may also be expressed in pheromone-sensing GRNs. However, the available data do not support that possibility. Previous studies showed that Gr5a-GAL4 do not overlap with markers for pheromone-sensing GRNs (*ppk23, ppk25*, and *fru*^{M}) (Lu et al., 2012; Thistle et al., 2012), and that *Gr5a*$^{+}$ GRNs did not respond to male pheromones (Lacaille et al., 2007). Furthermore, disruption of *Gr5a*$^{+}$ function does not decrease aggression when the aggression-promoting resource is females instead of food (Figure S8a), nor does it produce any effect on courtship or social behaviors (Fan et al., 2013; Lone and Sharma, 2012). Finally, disruption of *Gr5a*$^{+}$ GRNs function decreases aggression in the presence of sucrose (Figure S8c). Taken together, these data strongly argue against the possibility that the requirement for *Gr5a*$^{+}$ GRNs in aggression on food is due to a role in pheromone rather than sugar detection.

Aggression in flies is typically considered to be “territorial” (Chen et al., 2002; Hoffmann and Cacoyianni, 1990). However, there is a difference between the defense of a territory containing a particular resource, and the defense of the resource itself: a bird may defend a nest, or defend a larger area in which the nest is located. The available data do not distinguish between the two in the case of *Drosophila*. We observe that although single flies exhibit an innate attraction to food, in the presence of another male, they spend more time just outside the perimeter of the food area. Correspondingly, most fighting occurs in the perimeter

surrounding the food area. This “doughnut” effect is most apparent when the food patch becomes larger than 20mm in diameter; in smaller-diameter arenas, fighting occurs throughout the food patch.

These observations are consistent with (but do not prove) the idea that when the area of the food patch exceeds a certain size, flies adopt a “perimeter defense” strategy. Since such a strategy is the most energetically efficient way for a fly to prevent occupancy of a large food patch by its competitor, these results suggest that aggression in flies may indeed involve territorial defense. Nevertheless, we cannot formally exclude the possibility that flies fight at the patch perimeter simply because they prefer to occupy this area.

Taken together, our experiments show that food promotes aggression in flies in a manner that is not simply an indirect consequence of arousal, aggregation on food, or a general increase in social interactions. Flies increase and decrease the amount of aggression depending on the amount of food available, which is suggestive of competition over a limiting resource: aggression declines when the resource exceeds a certain threshold. The detection of this resource requires gustatory sugar receptor neurons that express *Gr5a*, consistent with the idea that it is the perceived caloric value of the resource that promotes aggression. Finally, flies exhibit a “perimeter defense” strategy, which is suggestive of a function for aggression to prevent the opponent from gaining access to a resource-rich territory. Together, these data offer new insights into the control of aggression in flies by food, which may apply to other species as well.

Materials and Methods

Behavioral Assays and Analysis

Behavioral assays were performed using 3-7 day old male flies that were raised in isolation. Group-housed flies were used in experiments shown in Figure 5, because group-housed male flies show female-induced aggression, unlike single-housed flies, which show a high level of baseline aggression even without females. In all experiments involving the Gr5a-GAL4 flies and their genetic controls, comparisons were made on equivalent genetic backgrounds. Most experiments were performed in a 40 mm x 50 mm behavior chamber previously described (Hoyer et al., 2008), or the new 70 mm x 70 mm chamber (Figure S4c) that allowed us to test different amounts of food. Briefly, two males were introduced into the chamber by gentle aspiration, recorded for 20 min, and behavioral data were extracted from the recorded videos using CADABRA software or directly from MATLAB. Temperature and humidity were kept around 25ºC and 40 - 50% R.H. and all experiments were performed around the activity peak of flies, either from 7 am to 3 pm or 7 pm to 3 am. As flies have to be able to see in order to fight, all experiments were performed using a ring-shaped strip of white LEDs to illuminate the behavioral chambers. From these analyzed movies, we extracted several parameters, such as position of flies with respect to food, frame by frame inter-fly distance, distance traveled, number of lunges performed, and number of circling behaviors performed. These parameters were manually checked to make sure that the tracking algorithm was reporting with high fidelity. For male-male one-wing

extensions, behavior was scored manually, as we found that CADABRA was unable to report an accurate count of male-male one-wing extensions. Thus, we used number of circling bouts instead of number of one-wing extensions to measure male-male courtship, except to show that food does not increase male-male courtship. All of the different chambers used can be seen in schematic drawings in Figure S4.

Fly Stocks and Rearing Conditions.

All fly stocks were reared in plastic vials containing yeast, corn syrup, and agar medium at 25°C, 60% humidity, and a 12-h light:12-h dark cycle. Newly eclosed males were reared either individually (single housing) or at 10 flies (group housing) per vial [2.4 cm (diameter) × 9.4 cm (height)] for 3 or 7 days before performing the behavioral assay. Wild-type Canton-S (CS) flies were used for all experiments, unless otherwise indicated. Gr5a-GAL4 flies were a gift from the John Carlson Lab. UAS-TNT and UAS-IMPTNT flies were acquired from Bloomington. UAS-Hid flies were a gift from Joel Levine Lab. UAS-Shi^{ts} flies were flies were a gift from obtained from the Gerald Rubin Lab (Pfeiffer et al., 2012). All transgenic flies used, such as the Gr5a-GAL4, UAS-TNT, UAS-IMP, UAS-Hid, UAS-nlsGFP UAS-Shi^{ts} were backcrossed for 6 generations into the CS background. All behavioral assays were performed using males carrying the wild-type X chromosome.

Statistical Analyses

Most of the behavioral data were nonparametrically distributed; thus, only

nonparametric tests were used to test for statistical significance. Mann-Whitney U tests (for pairwise comparisons) and Kruskal-Wallis analysis of variance (ANOVA; for comparisons among >2 groups) were applied. Significant difference among groups detected by Kruskal-Wallis ANOVA was analyzed using Dunn's *post hoc* test (with corrections for multiple comparisons) to identify groups with statistically significant differences. Two-way ANOVA was applied for comparisons among histograms.

Boxplots: lower and upper whiskers represent 1.5 interquartile range (IQR) of the lower and upper quartiles, respectively; boxes indicate lower quartile, median, and upper quartile and the cross indicates the mean. p values in all Figures represent Kruskal-Wallis one-way ANOVA followed by Mann-Whitney U tests with Bonferroni correction when there are more than two groups for comparison. p values are abbreviated using asterisks. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, N.S. (not significant): $p > 0.05$.

Figure 1

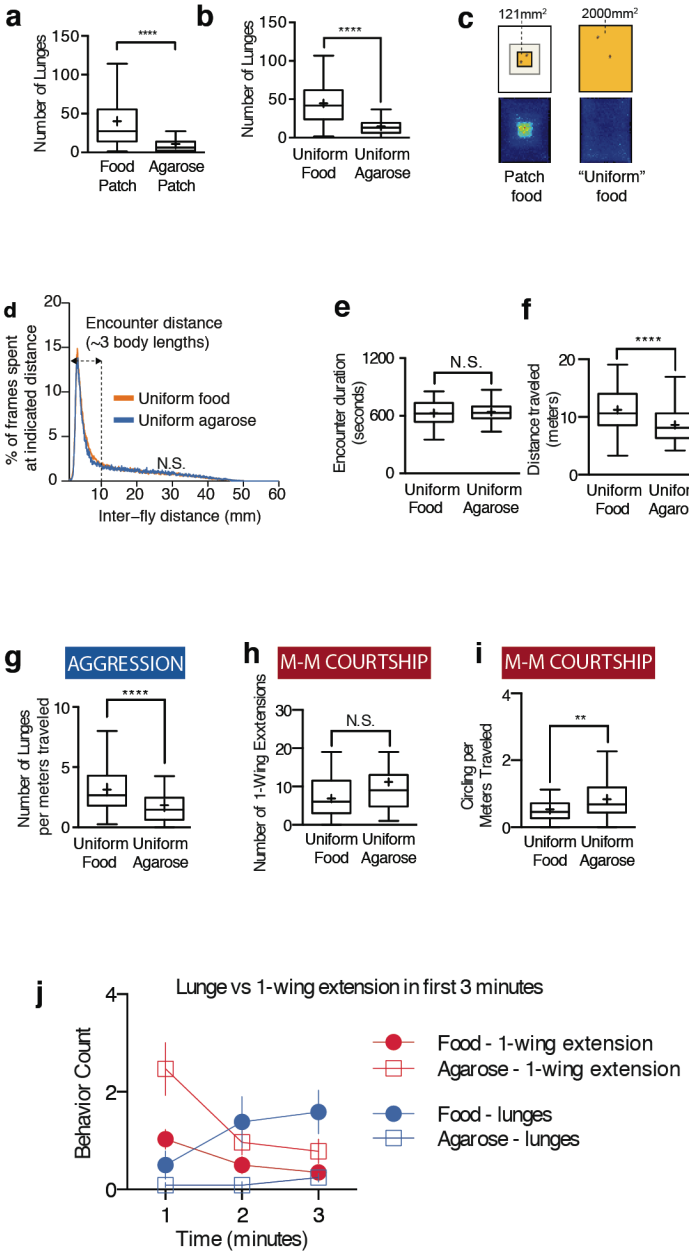


Figure 1. Food is necessary for normal levels of male-male aggression, but not male-male courtship, and its effects are independent from locomotion or encounter duration.

(a) Flies performed more lunges during the observation period in the presence of a 22 x 22 mm food patch. n= 171, 92 male-male pairs tested for apple juice food

patch and agarose patch, respectively. (b) Flies performed more lunges in the presence of arena, which was entirely covered with food. n = 113 and 44, for uniform food and uniform agarose, respectively. (c) Top: Schematic diagram of the aggression assay arenas used. Left side shows the food patch configuration and right side shows the uniform food configuration. A pair of male flies is illustrated at scale for comparison. Bottom: Position heat map shows the average amount of time flies spend in a particular position in the arena. The data shown are averages of multiple pairs of flies (same sample numbers as Figures 1a and 1b). It uses a red-blue color map from MATLAB where deep red is high frequency (60 frames, which is roughly 2 seconds, are the deepest-red) and blue is 0. Every subsequent position heat map is presented in the same manner. On the left, flies are attracted to the patch of food, while on the right, the uniform food does not lead to attraction to a specific spot in the arena. (d) Uniform food does not change the amount of time flies spend at various distances from each other. The inter-fly distance histogram shows amount of time flies spend (y-axis) at a given distance from each other (x-axis). The distribution is not affected by the presence of food (1-way ANOVA). There is a very prominent peak around 3-4 mm, which ranges from 2 mm (less than 1 body length of flies) to 10 mm (3-4 body lengths), and accounts for around 50% of the 20-minutes assay. The area under the curve from 0 to 10mm is hereafter referred to as “encounter duration.” The trace is the median trace from 72 and 44 male-male pairs for food and agarose, respectively. (e) Uniform food does not increase encounter duration. Assay is 20 minutes long (1200 seconds). Same number of samples as Figure 1d. (f) Locomotion (distance

traveled) in a pair of flies is increased in the presence of food. Same number of samples as Figure 1d. (g) Normalization of aggression by locomotion by dividing the number of lunges by travel distance shows that food significantly increases aggression. Same number of samples as Figure 1d. (h) Number of one-wing extensions is not changed by the presence of uniform food. Manually-scored data consisting of n = 17 and 18 pairs for food and agarose conditions, respectively. (i) Normalization of courtship (number of circling bouts) by locomotion shows that food decreases male-male courtship. Same number of samples as Figure 1d. (j) In the first three minutes, food progressively increases aggression (blue circle). In contrast, one-wing extension decreases (red circle). In the absence of food, lunges do not increase or decrease (blue box); courtship decreases (red box). See Table S1 for statistics. Manually-scored data of lunges and 1-wing extensions. n = 33 and 33 for food and agarose conditions for lunges. n = 34 and 31 for food and agarose conditions for one-wing extensions.

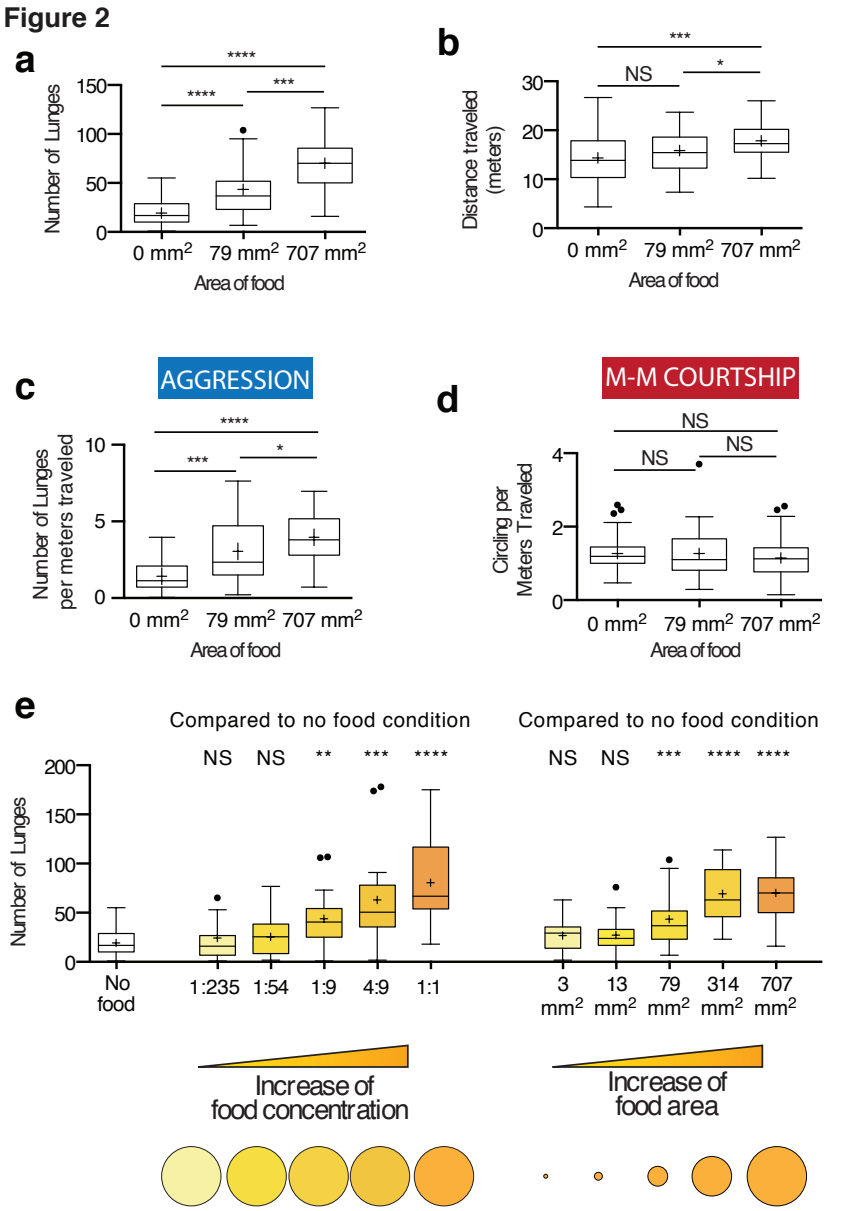


Figure 2. Flies measure the level of total nutrients to increase the level of aggression, rather than the area of food.

(a) Aggression increases as the size of food patch increases. See Figure S4 for

schematic diagrams of the arena used. n = 41, 39, and 52 male-male pairs for 0, 79, 707 mm^2, respectively. Same pairs are further analyzed for Figures 2b-d. (b) Locomotion also increases in some cases (0 vs. 707 mm^2) as the size of food increases. (c) Aggression normalized by locomotion is significantly increased in the presence of food. (d) Male-male courtship normalized by locomotion is not changed by the presence of food. (e) Left: Increasing the concentration of food while keeping the size of food constant (707 mm^2) increases aggression. Right: Increasing the size of food while keeping the concentration constant also increases aggression. The concentration-dependent increase in aggression is quantitatively similar to the size-dependent increase in aggression. The absolute nutritional content remains the same between the left and the right (1:235 = 3mm^2, 1:54 = 13mm^2, etc). Some of the data in E are the same as those used in A, and are replotted here for comparison purposes. n = 41, 22, 16, 29, 28, 31, 36, 37, 39, 27, and 52 male-male pairs from left to right.

Figure 3

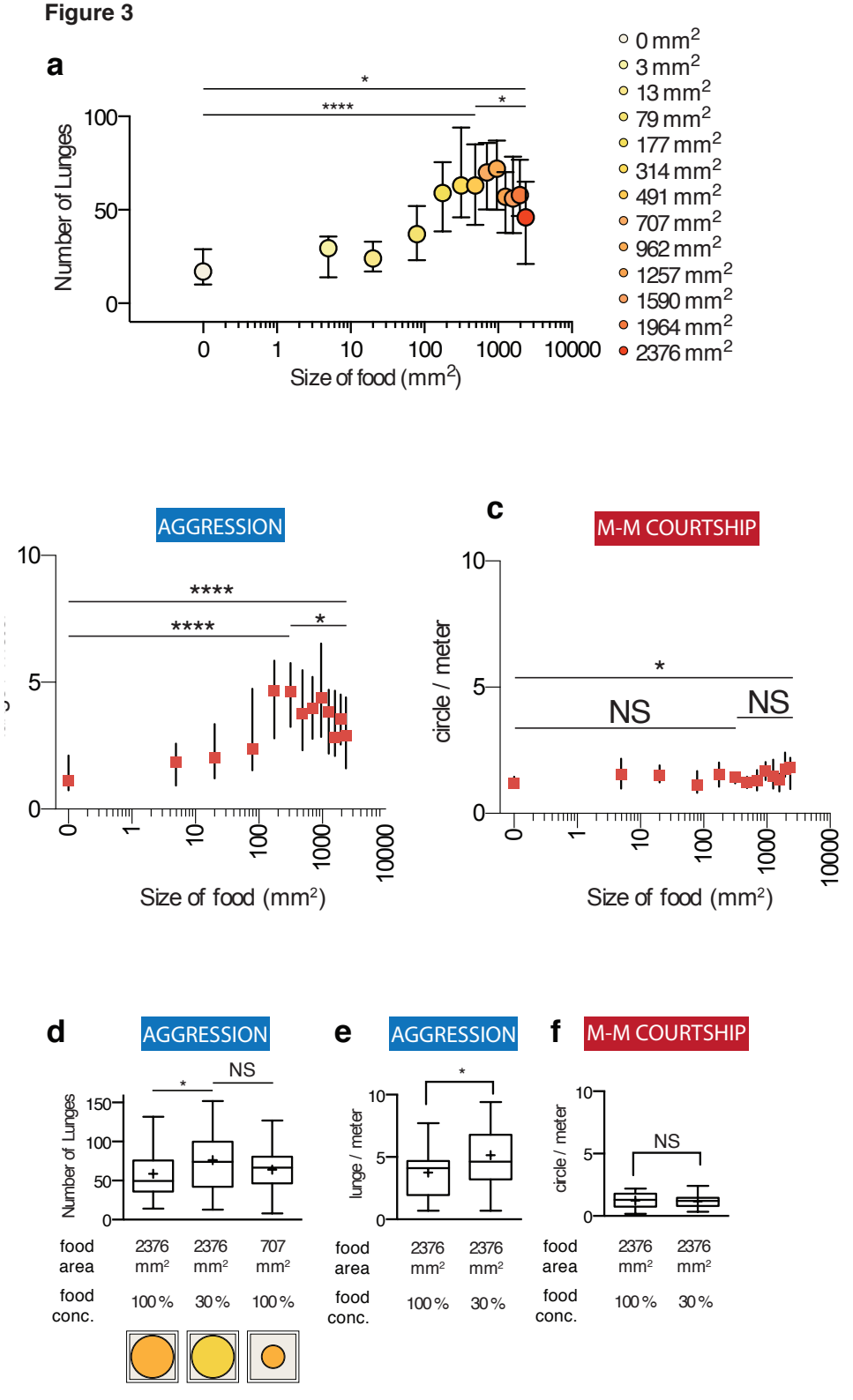

a
Number of Lunges
Size of food (mm²)
0 mm²
3 mm²
13 mm²
79 mm²
177 mm²
314 mm²
491 mm²
707 mm²
962 mm²
1257 mm²
1590 mm²
1964 mm²
2376 mm²
AGGRESSION
M-M COURTSHIP
c
circle / meter
NS
d
e
f
lunge / meter
food area
food conc.
100 %
30 %

Figure 3. Flies decrease the level of aggression as the availability of food resource increases.

(a) The relationship between aggression (y-axis) and the amount of food (x-axis). Aggression initially increases from 0mm^2 to 707mm^2, and decreases as the size of food increases further. In particular, aggression observed with the largest size tested (2376mm^2) is significantly lower than 707mm^2, after correcting for multiple comparisons. Some of the data are the same as those used Figure 2, and are replotted here for comparison purposes. n > 28 male-male pairs for each condition tested. Pairs are further analyzed for Figures 3b and 3c. (b) Aggression normalized by locomotion shows the same initial increase and subsequent decrease (See Table S3 for pair-wise comparison statistics). (c) Male-male courtship normalized by locomotion shows no increase or decrease (See Table S4 for statistics). (d) The decrease in aggression seen in the largest food patch tested (left, 2376mm^2) can be reversed by decreasing the concentration of food to 30% (middle). Calorically, this condition is equivalent to 707mm^2 food patch with 100% concentration of food (right) and the amount of aggression is indistinguishable. The 707mm^2 food patch data replotted for comparison purposes. n = 32, 31, 86 male-male pairs from left to right. (e) The increase in aggression by dilution of food is significant after normalization for locomotion. n = 32, 31. (f) There is no change in courtship caused by the dilution of food. n = 32, 31.

Figure 4

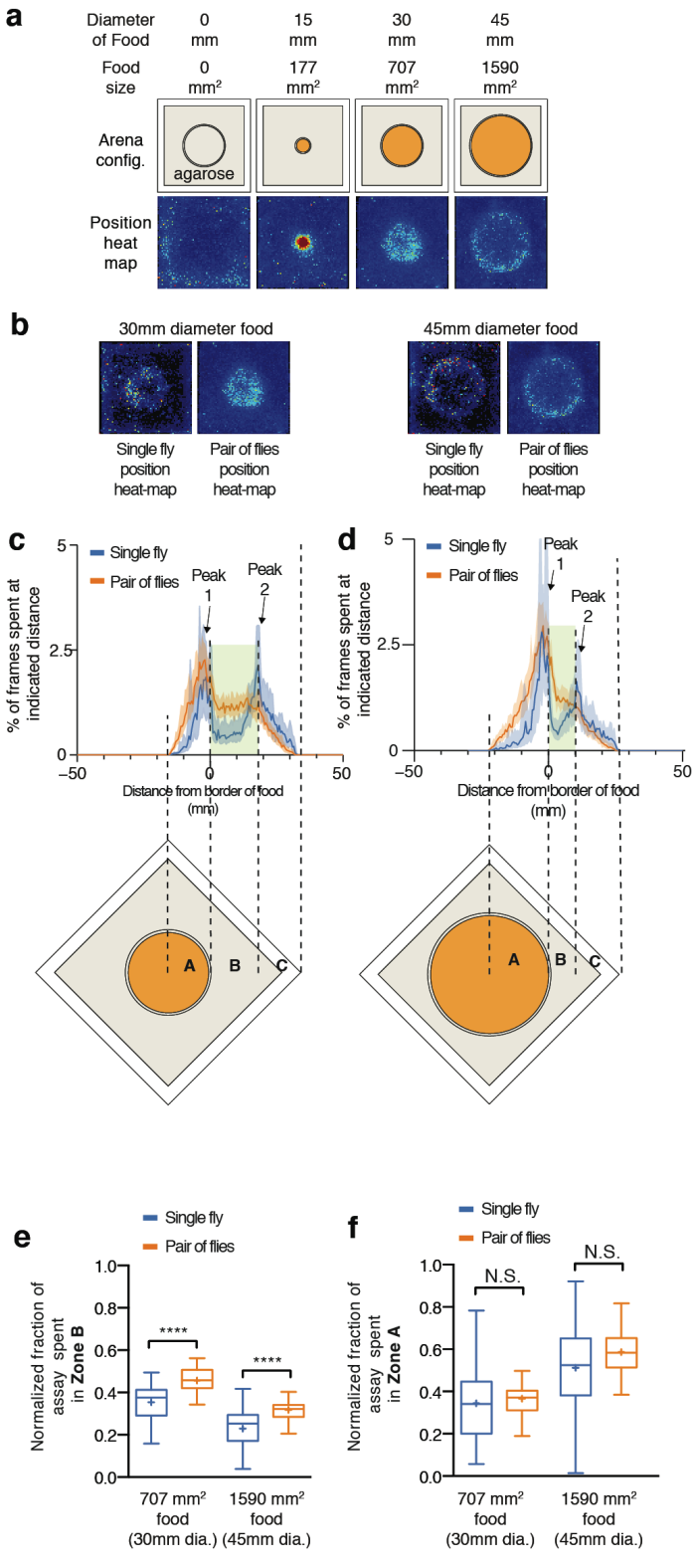

a
Diameter of Food
0 mm
15 mm
30 mm
45 mm
Food size
0 mm²
177 mm²
707 mm²
1590 mm²
Arena config.
agarose
Position heat map
b
30mm diameter food
45mm diameter food
Single fly position heat-map
Pair of flies position heat-map
Single fly position heat-map
Pair of flies position heat-map
c
d
Single fly
Pair of flies
Peak 1
Peak 2
% of frames spent at indicated distance
Distance from border of food (mm)
A
B
C
e
f
Normalized fraction of assay spent in Zone B
Normalized fraction of assay spent in Zone A
****
N.S.
707 mm² food (30mm dia.)
1590 mm² food (45mm dia.)

Figure 4. Flies display territorial behavior.

(a) Top row: Schematic diagrams show the arenas with different size of food being used. Bottom row: Position heat-map of a pair of flies presented with different sizes of food. The heat-maps display two features: 1) flies spend a lot of time on top of food, and 2) they spend a lot of time near the border of the food area. n = 41, 29, 86 and 41 male-male pairs from left to right. (b) Position heat map compares the distribution of flies on 30mm- and 45mm-diameter food when there is only 1 fly in the arena (left), and when there are two flies (right). 2-fly data from one experiment are individually averaged. n = 30 and 52 for 30mm-diameter food, single and pairs of flies, respectively. n = 25 and 41 for 45mm-diameter food, single and pairs of flies, respectively. The pairs are further analyzed in Figures 4c – 4f. (c and d) These histograms show the amount of time that flies spend at different distances from the border of 30mm (c) food and 45mm (d) patch. The schematic diagrams of the behavioral setups are overlaid for visualization. Briefly, the x-axis is aligned so that 0 denotes the border of food patch, while negative values indicate the distance inward from food border (inside the food patch), and positive values indicate the distance outward from the food border (outside of food patch). The blue line denotes when there is a single fly in the arena, while the orange line denotes when there is a pair of flies. Lines indicate the median, while the shaded area denotes the interquartile range. (e) Presence of another fly increases the amount of time that flies spend in Zone B (“interaction zone”) for both 30mm and 45mm food patches. (f) Presence of another fly does not change the amount of time that flies spend on the food patch (Zone A).

Figure 5

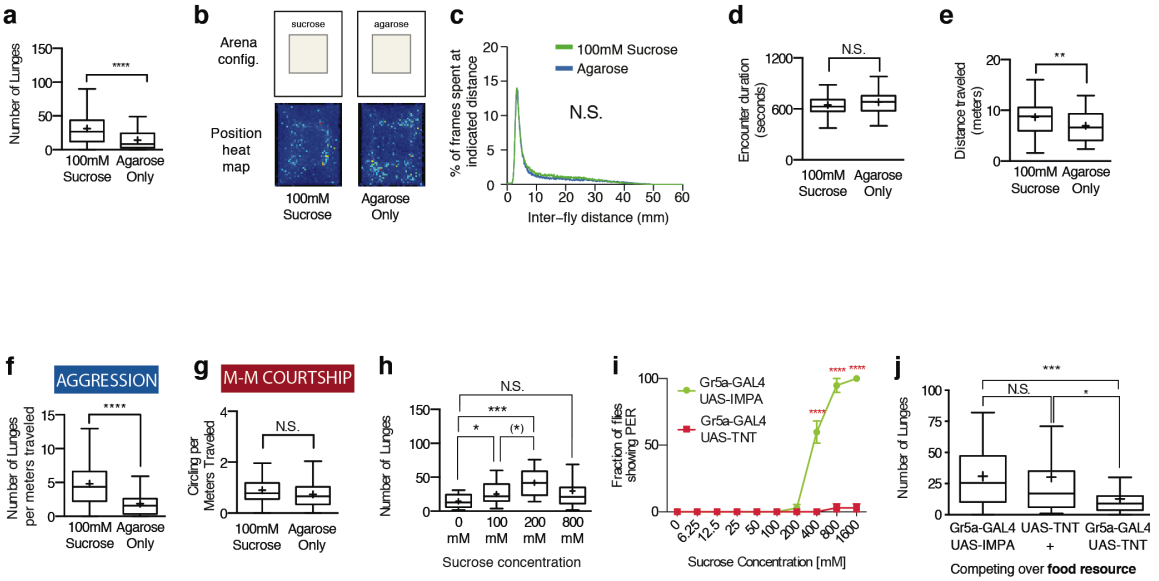


Figure 5. Flies use sweet-sensing *Gr5a*$^{+}$ GRNs to detect the concentration of sucrose in the food, and tune the level of aggression accordingly.

(a) 100 mM sucrose is sufficient to increases aggression. (b) Sucrose does not cause attraction, as it does not lead to an apparent change in the position heat map. n = 100 and 60 for 100 mM sucrose and agarose, respectively. Pairs are further analyzed from Figures 5b-5g. (c) Presence of sucrose does not change the amount of time that flies spend near each other. (d) Encounter duration does not change in the presence of sucrose. (e) Sucrose increases locomotion. (f) Sucrose increases the number of lunges per meters traveled, which implies that the increase in aggression is not merely due to increased locomotion. (g) Sucrose does not change the number of circling per meters traveled. (h) Changing sucrose concentration increases and decreases aggression. The level of aggression is increased from 0 to 200 mM, but becomes indistinguishable from no food condition

at 800 mM. (*): 100 to 200 mM difference is significant when individually compared ($P<0.05$), but not after corrections for multiple comparisons. n = 32, 23, 10 and 26 from left to right. (i) Inhibiting the sugar-sensing *Gr5a*$^{+}$ GRNs by expressing TNT decreases sucrose sensitivity (n = 3 and 3 for both genotypes. Each replicate has 10 male flies to calculate fraction of responders). (j) Inhibiting the sugar-sensing *Gr5a*$^{+}$ GRNs by expressing TNT decreases food-promoted aggression compared to genetic controls. n = 36, 41, and 32 from left to right.

Supporting Information Legends

Figure S1

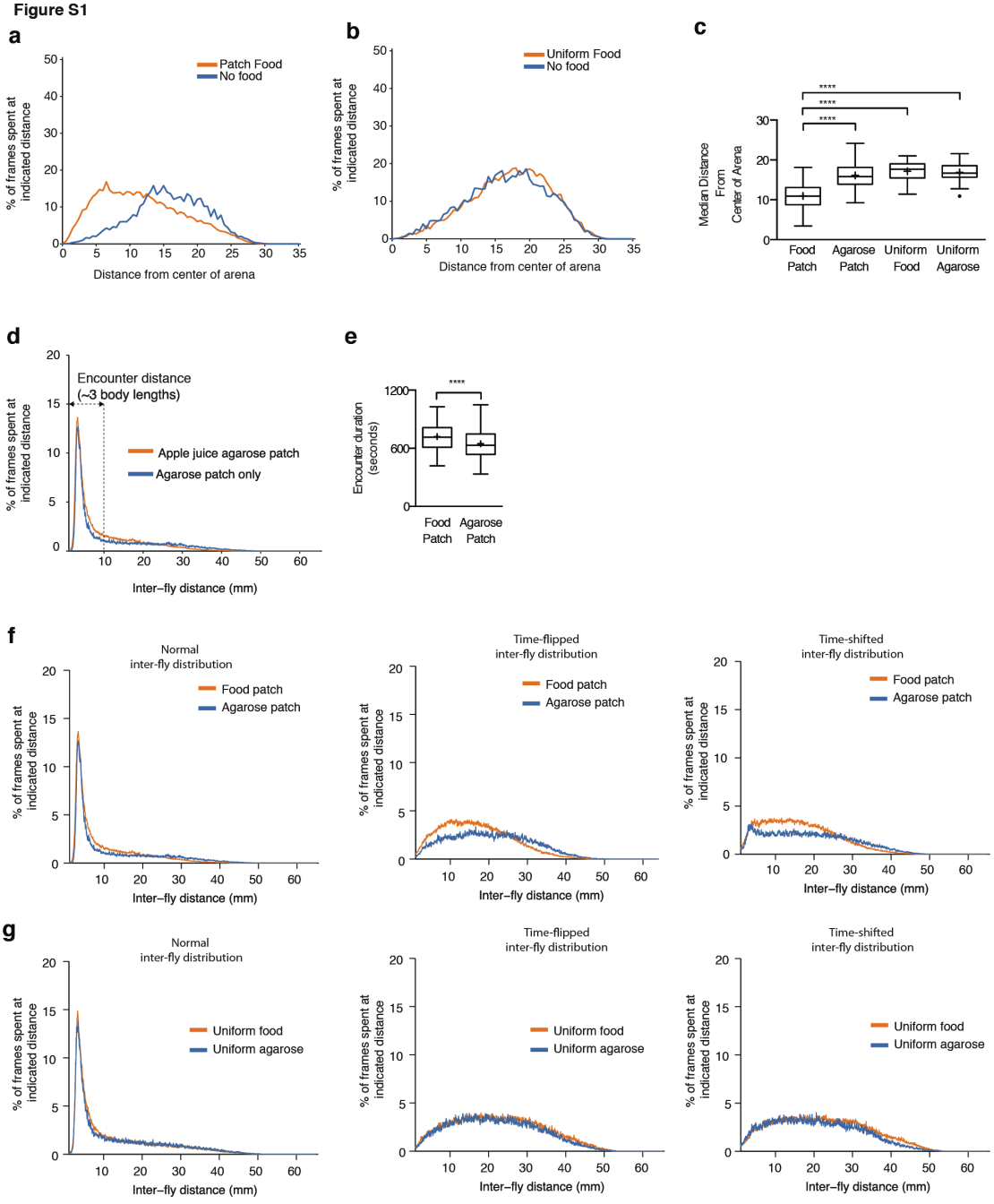


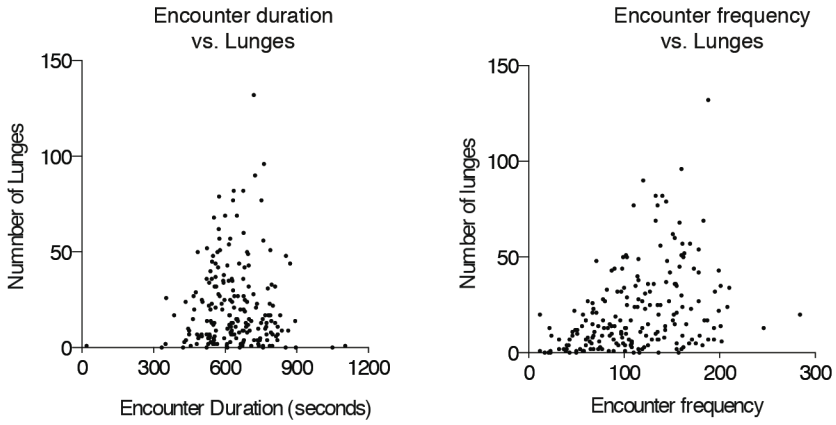
Figure S1. Proximity between two male flies is changed by the presence of a small food patch, but not by uniform food.

(a) In the presence of a small food patch, there is clear attraction to the center of the arena. n = 171 and 92 for food patch and agarose patch, respectively. n = 72 and 44 for uniform food and uniform agarose, respectively. The pairs are further analyzed for all of Supplemental Figures 1. (b) In the presence of food, which covers the surface of the arena uniformly, there is no change in the distribution of the flies with respect to the center of the arena. (c) Quantification of the data in (a) and (b): Median distances from the center of the arena are changed in the presence of a small food patch. (d) Inter-fly distance histogram shows that the presence of a small food patch slightly changes the distribution compared to the absence of food. (e) Sum of the encounter (inter-fly distance < 10mm) duration shows that the presence of a small patch of food slightly increases the amount of time flies spend within 10mm of each other. (f) Left: Same data as (d) replotted for comparison. Middle: Shows the same data as Left after transformation of the position of one fly with respect to time by flipping the order (first frame becomes last frame and vice versa). Transformation shows that flies are naturally attracted to the center of the arena but the prominent encounter peak is not present, suggesting that the peak depends on the coordinated positioning of two flies. Right: Shows the results of similar transformation as Middle, but instead of flipping the order, 1000 frames were added to shift one fly's position with respect to time. (g) Left: Same data as Figure 1D replotted for comparison. Middle and Right: Transformation as performed in (f) shows that the presence of uniform food does

not change the position of flies, and that the prominent peak in inter-fly distance histogram is likely due to the natural interaction distance of flies.

Figure S2

a



	Encounter duration	Encounter frequency
Pearson *r*	0.01830	0.3649
R^2	0.0003349	0.1332
P value	0.7950 (NS)	< 0.0001 (****)

Figure S2. Encounter duration is an independent measure of aggression.

(a) Encounter duration, the amount of time flies spend within 10mm of each other, shows no correlation (*r* = 0.018) with the number of lunges. Most of the points lie near 600 seconds (50% of the assay), regardless of the number of lunges observed. n = 204 x, y pairs. (b) Encounter frequency, the number of times flies come within 10mm of each other, shows a weak correlation (*r* = 0.365) with the number of lunges.

Figure S3

Aggression (lunges) and courtship (circling) are linearly correlated to locomotion.

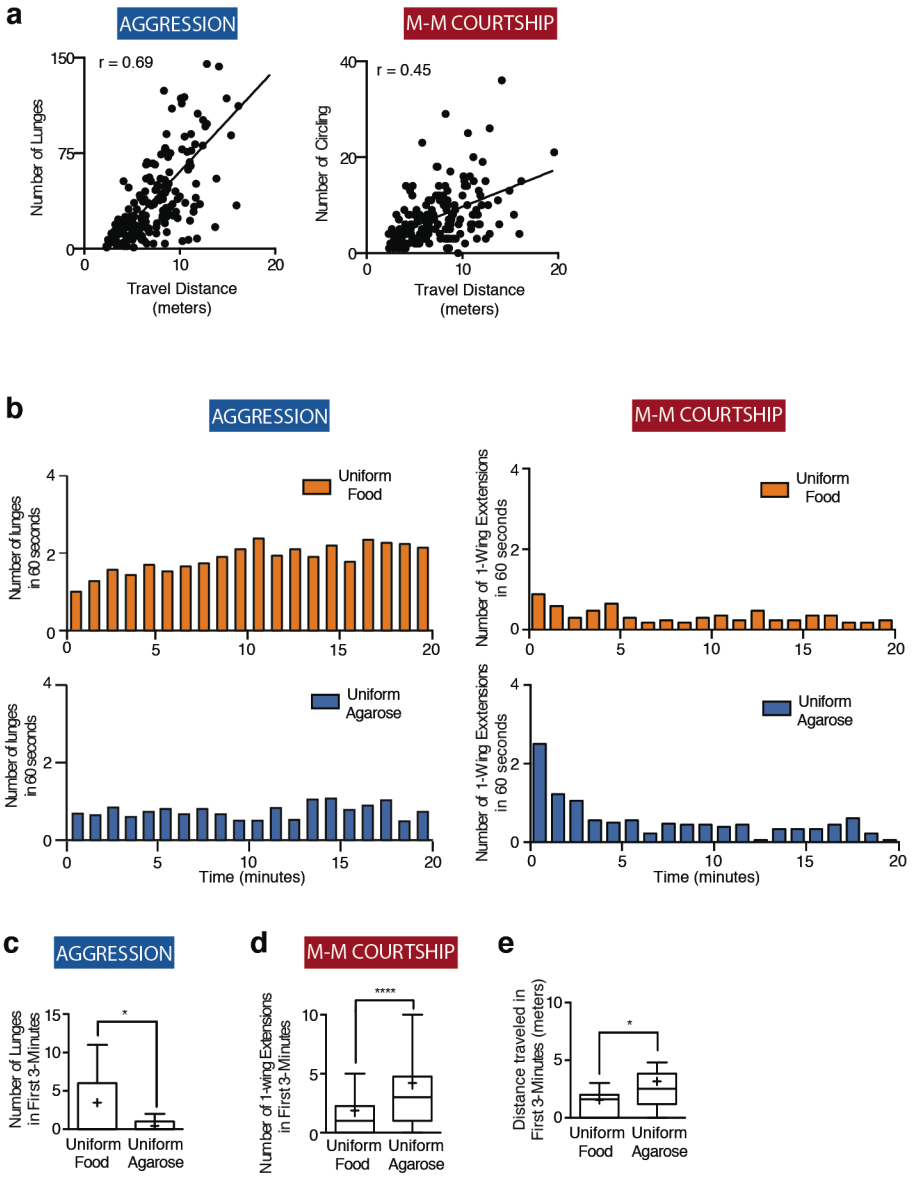


Figure S3. Food promotes aggression and not courtship.

(a) Left: Aggression (number of lunges, y-axis) is linearly correlated with locomotion (r = 0.69, travel distance in meters on x-axis). Right: Courtship (number of circling) is linearly correlated with locomotion (r = 0.45). n = 171 male-male pairs. (b). Behavioral choice between male-male courtship and male-male

aggression develops in the first three minutes of the assay and remains stable. Left: Aggression increases slightly over time in the presence of food (orange). No change is observed in the absence of food (blue). n = 113 for uniform food and 44 for uniform agarose. Right: Male-male courtship (one-wing extension) decreases slightly over time in the presence of food (orange) and without food (blue). One-wing extension data were manually scored. n = 18 and 17 for uniform food and agarose, respectively. (c) Presence of food increases aggression in the first three minutes of the assay. Manually-scored lunges for male-male pairs, n = 33 and 33 for food and agarose conditions. (d) Presence of food decreases male-male courtship (one-wing extensions) in the first three minutes of the assay. Manually-scored one-wing extensions for male-male pairs, n = 34 and 31 for food and agarose conditions for one-wing extensions. (e) Presence of food decreases locomotion in the first three minutes of the assay. n = 34 and 31 for food and agarose.

Figure S4

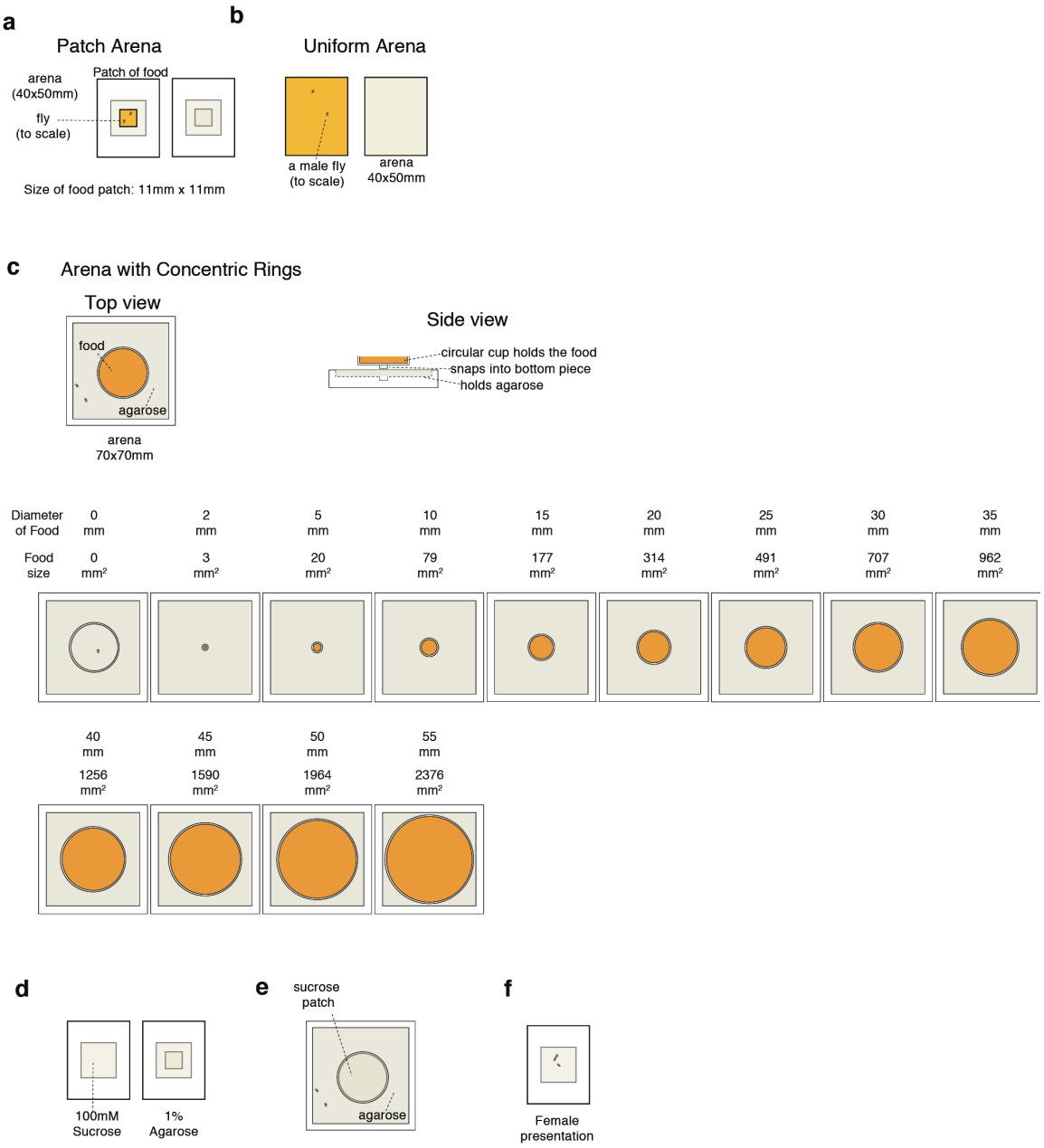


Figure S4. Schematic diagrams of all of the arenas used in behavioral assays. (a) Patch arena: 11mm x 11mm food patch is used and compared with agarose. Surrounding the food patch, there is an area with agarose. The arena is 40mm x 50mm. (b) The uniform arena has the entire surface covered with either food or

agarose. (c) An arena with concentric rings allows for testing of multiple sizes of food with diameters. The food patch is surrounded by agarose, which is surrounded by a small plastic base. The entire arena is 70mm x 70mm. (d) Experiments with the sucrose patch were performed with either sucrose or agarose in a 22mm x 22mm square area in the middle of the arena. (e) Experiments testing different sucrose concentrations (0, 100, 200, 800 mM) were performed with 707mm^2 patch of sucrose. (f) Experiments testing female-induced aggression were performed with 40mm x 50mm arena, with a dead female on top of an agarose patch in the middle.

Figure S5

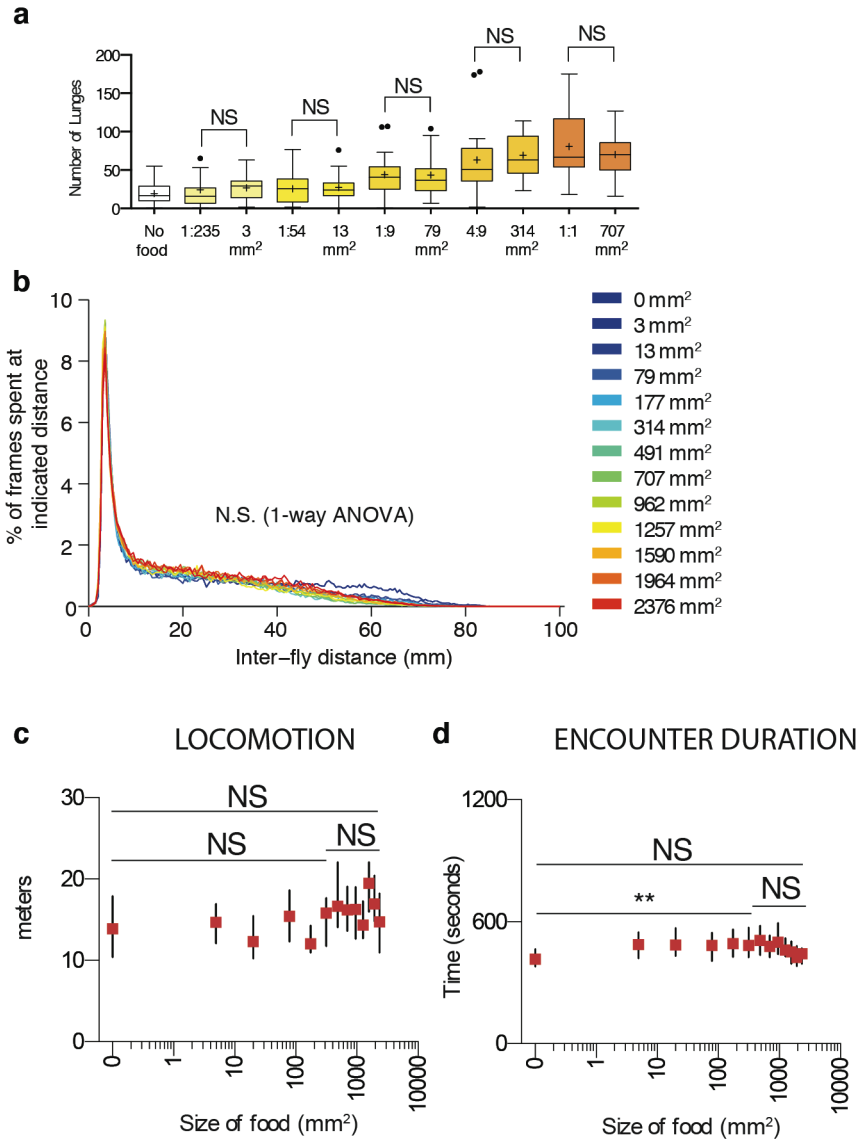


Figure S5.

(a) The absolute amount of food, rather than concentration or area of food, determines the level of aggression (1:235 dilution of food with 707mm^2 area is equivalent to a 3 mm^2 food patch, etc). Every dilution–size pair is statistically indistinguishable from the other condition. The data are replotted from Figure 2e for comparisons. (b) Inter-fly distribution shows the pattern of inter-fly distance

does not change over 13 different sizes of food patch ranging from 0 to 2376mm^2 (1-way ANOVA). n > 28 for all conditions. (c) Locomotion shows little to no change as the size of food changes from 0 to 2376 mm^2. See Table S2 for details. n > 28 for all conditions. (d) Encounter duration shows no change as the size of food changes from 0 to 2376 mm^2. See Table S5 for statistics. n > 28 for all conditions.

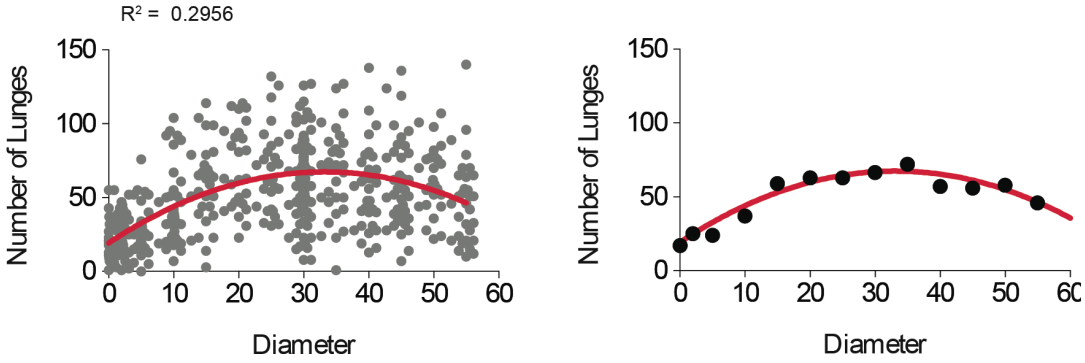
Figure S6

a

$$Y_i = \beta_0 X_i^2 + \beta_1 X_i + \beta_2 + \varepsilon_i$$

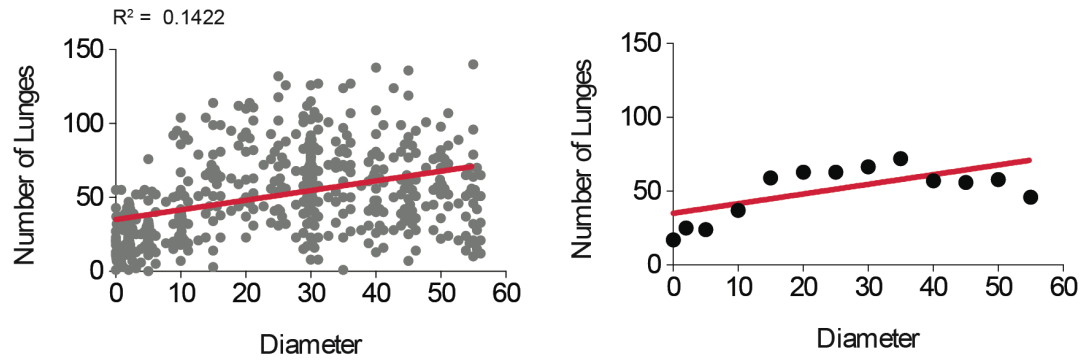
$$\partial Y / \partial X_i = 2\beta_0 X_i + \beta_1$$

b



Coefficient	Statistical significance	99% confidence interval
$\beta_0 = -0.044$	P < 0.001	[-0.055, -0.033]
$\beta_1 = 2.9$	P < 0.001	[2.3, 3.5]
$\beta_2 = 20$	P < 0.001	[14, 26]

c



Coefficient	Statistical significance	95% confidence interval
$\beta_0 = 0.65$	P < 0.001	[0.51, 0.80]
$\beta_1 = 30$	P < 0.001	[31, 39]

Figure S6. Aggression shows biphasic response to the amount of food.

(a) Functional form being tested for curve-fitting analysis. (b) Curve-fitting the quadratic function of the form in (a) shows that there is an increasing and decreasing pattern. Left: Scatter plot of the experimental data (n = 493). x-axis is diameter of food, and y-axis is number of lunges. Right: Each dot represents the median of the data plotted left. Red line is the resulting curve from the regression analysis. Table shows the coefficients from the ordinary least squares (OLS). Statistical significance values represent the t-test against the null-hypothesis that the coefficient is zero. (c) Regression to a linear function does not fit the data as well as a quadratic function, which increases and decreases. Same experimental data are replotted here for comparison purposes. Left: Overlay of scatter plot with the linear function from the OLS. Right: Overlay of medians plotted with the linear function. Table shows the coefficients from the OLS.

Figure S7

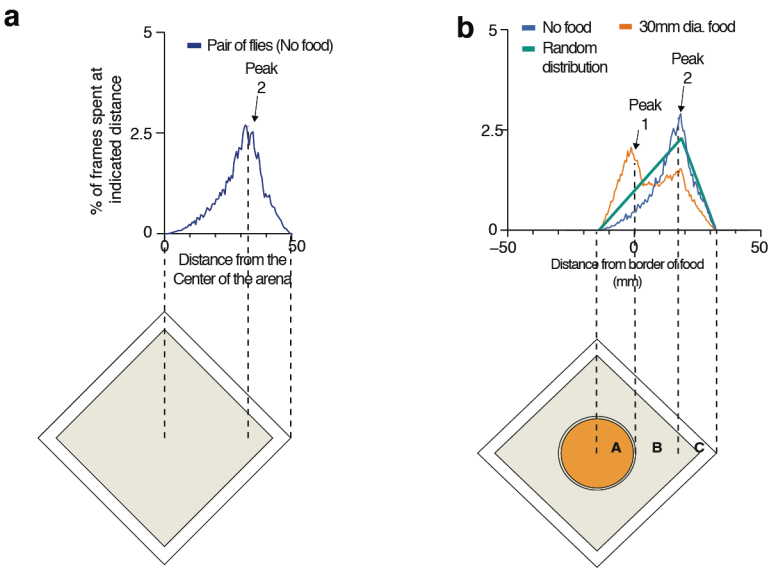


Figure S7. Overlay of Figure 4C and 4D onto the arena.

(a) In the absence of any food patch, fly position histogram shows a peak roughly 15-20mm from the edge of the arena. (b) Comparison of 30mm diameter of food patch (orange) to no food patch (blue, same data from Figure S7a replotted for comparison) and random distribution (teal). There is a clear difference in the distribution of fly positions between the arenas with the food patch vs. no food patch. The random distribution, expected if flies uniformly occupied the arena, shows that it is qualitatively similar to no-food condition, but very different from the arena with a 30mm food patch.

Figure S8

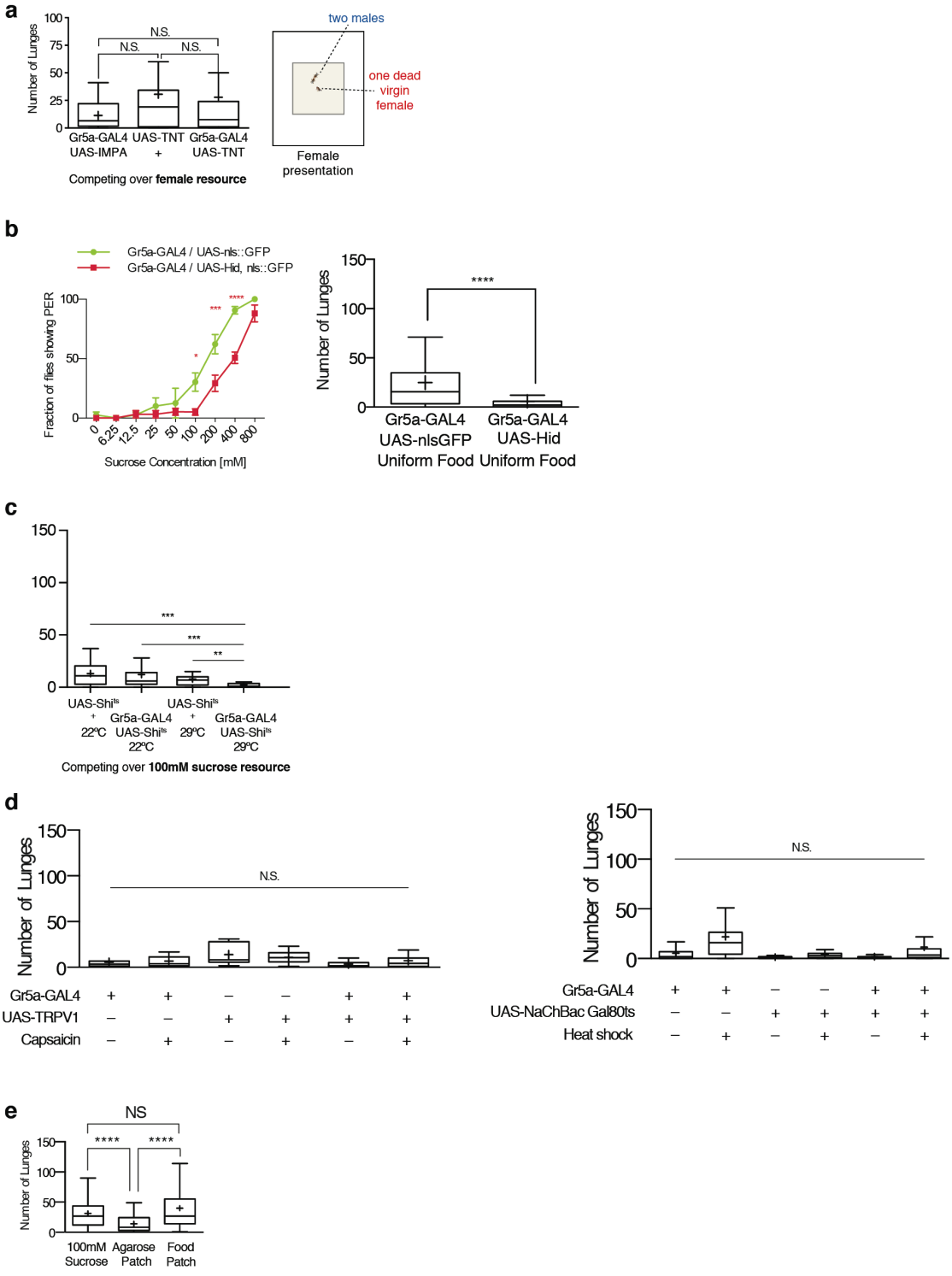

a
Number of Lunges
N.S.
N.S.
N.S.
Gr5a-GAL4 UAS-IMPA
UAS-TNT +
Gr5a-GAL4 UAS-TNT
Competing over female resource
two males
one dead virgin female
Female presentation
b
Gr5a-GAL4 / UAS-nls::GFP
Gr5a-GAL4 / UAS-Hid, nls::GFP
Fraction of flies showing PER
Sucrose Concentration [mM]
Number of Lunges
****
Gr5a-GAL4 UAS-nlsGFP Uniform Food
Gr5a-GAL4 UAS-Hid Uniform Food
c
***
***
**
UAS-Shi^ts + 22ºC
Gr5a-GAL4 UAS-Shi^ts 22ºC
UAS-Shi^ts + 29ºC
Gr5a-GAL4 UAS-Shi^ts 29ºC
Competing over 100mM sucrose resource
d
Number of Lunges
N.S.
Gr5a-GAL4
UAS-TRPV1
Capsaicin
N.S.
Gr5a-GAL4
UAS-NaChBac Gal80ts
Heat shock
e
NS
****
****
Number of Lunges
100mM Sucrose
Agarose Patch
Food Patch

Figure S8. Activity in *Gr5a*$^{+}$ GRNs is necessary for food-promoted aggression, but not sufficient for normal levels of aggression.

(a) Inhibition of *Gr5a*$^{+}$ GRNs by expression of UAS-TNT does not affect the level of aggression in the presence of females. n = 26, 32, 32 from left to right. Schematic figure shows the assay performed with a freeze-killed virgin female presented in the middle of the arena, partially embedded in agarose to prevent copulation. Two male flies are scored for aggressive behavior. (b) Inhibition of *Gr5a*$^{+}$ GRNs by expression of UAS-Hid decreases sucrose-response (left, n = 4 and 4 for both genotypes. Each replicate has 10 male flies to calculate fraction of responders) and aggression in the presence of uniform food (right,n = 40 and 40 male-male pairs for both genotypes). (c) Silencing of *Gr5a*$^{+}$ GRNs by expression of UAS-Shi^{ts} decreases aggression on 100 mM sucrose (n > 26 for all conditions). d) Activation of *Gr5a*$^{+}$ GRNs by expression of UAS-TRPV1 and UAS-NaChBac, tub-Gal80ts fails to increase aggression in the absence of food. n = 8, 12, 6, 8, 31, 34 for UAS-TRPV1 and 21, 31, 18, 35, 21, 49 for UAS-NaChBac Gal80ts. (e) Sucrose patch increases aggression to a level comparable to a food patch. n > 84 for all three conditions tested.

Chapter 3

A COMBINATORIAL CHEMOSENSORY CODE CONTROLS INTER-MALE AGGRESSION

Introduction

As I have reviewed in the Introduction chapter, males of many vertebrate and invertebrate species compete over females. In *Drosophila*, although it has been suggested that the presence of females increases aggression in many studies (Chen et al., 2002; Hoffmann and Cacoyianni, 1990; Skrzipek et al., 1979; Yuan et al., 2014). Nevertheless, these studies were performed with food as well as females, which made it unclear whether females alone could increase aggression. Furthermore, these studies did not show whether females increased attraction, and if so, whether the increase in aggression was due to the changes in encounter duration between males. Finally, although female detection has been extensively studied in the context of male-female courtship, much less was known about how males detect the presence of females as a resource, over which to compete.

In this chapter, I will present results that characterize how females promote male-male aggression in *Drosophila*. I show that females can increase aggression in the absence of food, and that this effect on aggression does not depend on changes in encounter duration or locomotion. Furthermore, I show that males use female-specific cuticular hydrocarbon (CH) pheromones to detect the presence of a mate resource. In addition, I show that these CH pheromones likely mediate their effects on aggression via *ppk23*$^{+}$ and *ppk25*$^{+}$ gustatory neurons, which are necessary and sufficient for male-male aggression in some contexts. I also found that male flies require the presence of both male and female CH pheromones in order to fight, as absence of either one abolishes male-male aggression,

suggesting a binary chemosensory code that determines male social behaviors. Finally, I show that newly-generated genetic tools may be useful in dissecting how the chemosensory binary code that I propose may work at the level of sensory circuits.

Males fight over females

It has been reported that females, when presented with food, can be an appetitive resource that promotes male-male aggression in *Drosophila* (Chen et al., 2002; Hoffmann, 1987; Hoffmann and Cacoyianni, 1990; Jacobs, 1960; Yuan et al., 2014). Since I showed that food presented alone is sufficient to increase aggression, it remained a possibility that the increase in aggression in the presence of food and females was due to food alone. Thus, I sought to test whether presentation of females compared to in the absence of food is sufficient to increase aggression in a pair of males (Figure 1a).

I found that presentation of a wild-type virgin female (freeze-killed and embedded in agarose to prevent copulation), but not a wild-type male (also freeze-killed and embedded in agarose in the same manner), was sufficient to promote aggression among two single-housed males (Figure 1b), even in the absence of food. The analysis software we used in studying male-male aggression has not been tested using this modified setup using presentation of a dead fly (Dankert et al., 2009). Introduction of females leads to quantitatively and qualitatively different male behaviors, such as increase in courtship behaviors that are occasionally

seen in male-male pairs such as one-wing extension or circling, as well as many new behaviors, such as copulation attempts and necrophilic copulation if female genitals are exposed. Therefore, I verified that the system is still able to reliably report the number of lunges seen in the presence of females by manually scoring the movies and comparing them with the QTRAK-CADABRA output. This analysis showed that our analysis software reports a highly accurate number of lunges compared to manually scoring (Figure 1c). Thus, I relied on our analysis software in all subsequent experiments to measure the number of lunges (i.e. amount of male-male aggression). Strikingly, the presentation of a female increased aggression not only in single-housed flies, but also in a pair of group-housed flies (Figure 1d), which normally do not fight in the presence of food alone (Hoffmann, 1990; Wang et al., 2008).

Males fight over females independent of effects on encounter duration

Since females, like food, are a resource, it is possible that females increase aggression by simply increasing the proximity between the two flies due to their attraction to females. Nevertheless, presentation of females compared to presentation of males did not significantly increase the encounter duration (amount of time flies spent within 10mm of each other) between male flies (Figure 1e and Figure 2f). These results suggest that the increase in aggression in the presence of females is not due to a nonspecific increase in proximity or interactions between males.

In order to further distinguish whether the effect of females to increase aggression was due to an indirect effect of females to attract males to one particular spot, we repeated the assays in a modified setup, where instead of one female or one male presented, 10 females or 10 males were presented, evenly distributed throughout the arena (Figure 2a). In the presence of 10 females, there was still a robust increase in aggression in the presence of females when compared to presentation of 10 males (Figure 2b). Furthermore, the increase was statistically indistinguishable from the presentation of 1 female (Figure 2b), suggesting that unlike food (Chapter 2, Figure 2), there is no apparent dose-dependent effect in female-induced male-male aggression.

Females increase general or social arousal among males

In addition to aggression, the presence of females seemed to increase general arousal, as it increased many behaviors in group-housed males. Group-housed males were mostly inactive in the presence of a dead male, but became much more active in the presence of females (Figure 2c). Since increase in locomotion is correlated with increase in any social behaviors, it is unclear whether the increase in locomotion was responsible for increase in social behaviors, or vice versa. As with food, the presence of females increased aggression disproportionately compared to locomotion, as normalizing the number of lunges by the locomotor activity still showed a robust increase (Figure 2d).

Unlike food, which I showed specifically increased aggression among two

males but not courtship toward each other, females profoundly increased both male-male aggression and courtship (summarized in Table 2e). Since females increase locomotion, courtship and aggression, it is not possible to distinguish whether females increase aggression as a result of increase in general or social arousal, or increase aggression independently.

Female pheromones are necessary for female-induced aggression

Previous studies have shown that *Drosophila melanogaster* males detect the presence of females by using female-specific cuticular hydrocarbon pheromones (CHs), such as 7,11-heptacosadiene (7,11-HD) and 7,11-nonacosadiene (7,11-ND), which are sufficient to produce courtship behaviors in male flies (Antony and Jallon, 1982; Ferveur, 2005; Jallon, 1984). In addition to these CH pheromones, which are detected by contact chemoreceptors, male courtship behavior is also modulated by vision (Krstic et al., 2013; Tompkins et al., 1982) and olfaction (Gailey et al., 1986; Grosjean et al., 2011; Jallon, 1984; Krstic et al., 2009; Kurtovic et al., 2007; van der Goes van Naters and Carlson, 2007; Wang et al., 2011). It is important to note that these studies examined the effect of sensory cues on female-directed courtship in males; thus, it is not clear which among these sensory cues, if any, play a role in female-induced male-male aggression.

Since CH pheromone plays a particularly important role in female detection in courtship (Antony and Jallon, 1982; Ferveur, 2005; Jallon, 1984; Thistle et al., 2012), I first tested whether female CHs similarly play an important role in female-

promoted aggression. To do this, I presented the males with females washed in hexane—a manipulation that washes away hydrophobic cuticular hydrocarbons (Savarit et al., 1999)—and compared them to control females, which were not washed with hexane.

These experiments showed that hexane-washed females do not promote male-male aggression, suggesting that female-specific CHs may be necessary for food-independent, female-induced male-male aggression (abbreviated as FIFIMMA, Figure 3a). Since washing females with hexanes involves soaking the entire female body in hexane, it is possible that there might be differences in visual cues or olfactory cues in the washed females. For instance, it is known that female-specific olfactory cues can activate olfactory receptor neurons (ORNs), such as *Or47b*$^{+}$ and *Or88a*$^{+}$ ORNs, in males, although the chemical identity of these volatile female pheromones is not known (van der Goes van Naters and Carlson, 2007). Thus, to test whether cuticular hydrocarbon contact pheromones are specifically involved, I used the females, which are genetically engineered to specifically ablate pheromone-producing cells (Billeter et al., 2009). When male pairs were presented with oenocyte-ablated, CH-less female (oe- females) compared to control females with normal pheromonal profile (oe+ females), they did not fight, suggesting that CHs are necessary for MMA (Figure 3b).

Female pheromones, or 7,11-HD, are sufficient to increase male-male aggression

The foregoing experiments show that FIFIMMA requires female-specific CHs, which are produced by oenocytes. Loss-of-function results in aggression experiments by themselves are difficult to interpret, since lower aggression often correlates with reduction in locomotion and courtship (Figure 2e). Thus, in order to assign a causal link between female-specific CH and female-induced male-male aggression, I sought to test whether female CHs are sufficient to increase male-male aggression.

To test whether female CHs can restore FIFIMMA in oe- females, I perfumed oe- females with female CHs by housing the oe- females with by themselves or with wild-type females overnight. When I presented these oe- females housed together with wild-type females, male flies fought, whereas in the presence of oe- females housed with other oe- females, male flies did not fight (Figure 3c). These experiments suggest that female pheromones are necessary and sufficient for FIFIMMA.

Female cuticles have multiple CHs, which are not present in male cuticles (Antony and Jallon, 1982; Antony et al., 1985; Jallon, 1984). Among these female-specific CHs, 7,11-HD is most potent as an aphrodisiac producing male courtship behaviors and is present in highest quantities (Antony and Jallon, 1982; Antony et al., 1985). Therefore, I tested whether application of synthetic 7,11-HD can restore FIFIMMA in oe- females, and found that 7,11-HD (dissolved in solvent) applied to oe- females is sufficient to increase male-male aggression compared to control oe- females perfumed with solvent (hexane, Figure 3d). Thus, 7,11-HD

along with the female body is sufficient to increase FIFIMMA, suggesting that the ability of female CHs to increase male-male aggression could be mimicked by 7,11-HD. Similar experiments were performed in parallel with 7,11-nanocosadiene (7,11-ND) which is another female-specific pheromone (Antony and Jallon, 1982; Antony et al., 1985; Jallon, 1984). Although more experiments are necessary, preliminary results showed that series of dilutions with 7,11-ND did not increase aggression, while 7,11-HD did (Supplemental Figure 1).

Next, I tested whether female visual cues were necessary for the ability of 7,11-HD to increase FIFIMMA. To do this, I applied oe- males, who are visually indistinguishable with wild-type males, with 7,11-HD. This also resulted in an increase in male-male aggression, suggesting that female visual cues are dispensable for FIFIMMA (Figure 3e). The level of aggression seen in the presence of perfumed males was indistinguishable from the level of aggression seen in the presence of perfumed females. These results suggest that 7,11-HD can act alone in the absence of female-specific cues.

Finally, I sought to test whether 7,11-HD alone is sufficient to increase male-male aggression by applying 7,11-HD to filter paper in the absence of any perfumed fly body. In the absence of any fly body, 7,11-HD did not increase courtship or aggression among males (n = 18, data not shown). These data suggest that female CHs are necessary and sufficient for FIFIMMA, and 7,11-HD perfumed on a fly body is sufficient for FIFIMMA, but 7,11-HD alone in the absence of any fly visual or olfactory cues cannot mimic FIFIMMA. These results

are in agreement with our laboratory's previous finding that 7,11-HD perfumed onto a live target oe+ male does not increase male-male aggression (Wang et al., 2011). Since 7,11-HD only seems to increase aggression when it is perfumed onto a dead oe- male or oe- female fly, context seems to be important for 7,11-HD to increase male-male aggression. Since 7,11-HD is a contact pheromone, which is detected by the gustatory system (Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014) these data suggest that the gustatory system may play a role in FIFIMMA, and other components present in an oe- male or oe- female are also required.

Or47b$^+$ ORNs are not necessary for female-induced male-male aggression

Before the recent discoveries regarding *ppk23*$^+$*/ppk25*$^+$*/fru*$^+$ GRNs' role in pheromone detection (Lu et al., 2014; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014), Or47b+ ORNs, which respond to male and female cuticular hydrocarbon extracts (van der Goes van Naters and Carlson, 2007), were candidate receptors neurons for detecting courtship-promoting cues. *Or47b*$^+$ ORNs co-express *fruM* and project to sexually dimorphic glomeruli VA1lm (Couto et al., 2005; Fishilevich and Vosshall, 2005), and they are used by males to find females (Root et al., 2008). Furthermore, they were also implicated in male-male courtship and other social behaviors (Lone and Sharma, 2012; Wang et al., 2011). These data suggested the possibility that *Or47b*$^+$ ORNs may also mediate female detection in the context of female-induced male-male aggression.

Therefore, I tested whether *Or47b*$^{+}$ ORNs were necessary for the effect of female to increase male-male aggression by expressing *UAS-Kir2.1* in these ORNs by using the *Or47b-GAL4* (Fishilevich and Vosshall, 2005). In the presence of dead females, both control males (*Kir2.1/+*) and *Or47b*-silenced males (*Or47b-GAL4/UAS-Kir2.1*) increased their level of aggression, compared to the control condition in the presence of dead males (Figure 4a). In addition, *Or47b* silencing did not seem to affect male-male aggression in the presence of food, although a trend toward increase was observed (Figure 4b). These data suggest that *Or47b*$^{+}$ ORNs do not play a role in FIFIMMA.

pickpocket23-expressing and _pickpocket25_-expressing GRNs may be necessary for male-male aggression in some contexts

The foregoing experiments with Or47b+ ORNs suggested that another class of sensory neurons mediates female-detection in the context of female-induced aggression. In addition to ORNs, male flies also use GRNs to detect sex-specific CH pheromones (Antony and Jallon, 1982; Jallon, 1984; Meunier et al., 2000). *ppk23*$^{+}$/*ppk25*$^{+}$/*fru*$^{+}$ GRNs mediate both male and pheromone detection (Lu et al., 2014; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014). *ppk23*$^{+}$ GRNs consist of two distinct populations of *fru*$^{+}$ GRNs, one that detects male pheromones, such as 7-P, 7-T and cVA, and another that detects female pheromones, such as 7,11-HD and 7,11-ND (Thistle et al., 2012). This suggested the possibility that *ppk23*$^{+}$ GRNs may play a role in both male and

female detection in the context of female-induced male-male aggression.

Nevertheless, the evidence for *ppk23*$^{+}$/*ppk25*$^{+}$/*fru*$^{+}$ GRNs in male and female CH detection was most compelling, as it was independently confirmed by multiple groups (Lu et al., 2012; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014). To test whether *ppk23*$^{+}$ GRNs are necessary for male-male aggression, I expressed *UAS-TNT* (one of the eight original tetanus toxin insertions originally described in [Sweeney et al., 1995]) and *UAS-Shibire*^{ts} (a temperature-sensitive mutant version of *Drosophila* dynamin) (Kitamoto, 2001) in these GRNs—manipulations that were shown to disrupt the activity of these GRNs (Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014)—then tested these flies in the presence of either food or females. It is important to note that the following experiments are preliminary, and should be interpreted with caution. The reasons are twofold: 1) Due to very large variability of these genotypes and the large number of comparisons being made, in some cases, only strong trends were observed without statistical significance. 2) In some cases, the control genotypes such as *UAS-TNT/+* and *GAL4/UAS-IMPA* (one version of the mutant inactive tetanus toxin insertions, which lack proteolytic activity) (Sweeney et al., 1995) did not fight at comparable levels. Furthermore, for experiments with *UAS-Shibire*^{ts}, there may have been GAL4-independent leaky expression of the effector, leading to a non-specific decrease in behavior. These issues could be resolved by either higher repetitions or by using other effectors such as *UAS-Hid* or *UAS-Kir2.1*. There are at least two *ppk23-GAL4* lines

described: one from the Barry Dickson lab (denoted “Dickson” ppk23) and another from the Kristin Scott lab (denoted “K. Scott” ppk23). *ppk25-GAL4* line comes from the Pikielny lab (denoted “Pikielny” ppk25).

First, I tested whether *ppk23*$^{+}$ GRNs or *ppk25*$^{+}$ GRNs are necessary for female detection in the context of aggression. Due to the large variability in the level of aggression, it was not possible to find statistical significance using *UAS-TNT* (Figure 5a, left). Using *UAS-Shibire*^{ts}, it was possible to find significance in the case of *ppk25-GAL4* (Figure 5a, right). Most parsimonious explanation is that only *ppk25*$^{+}$ GRNs are required in the context of FIFIMMA. Nevertheless, given the strong trend toward decrease in both ppk23 lines with *UAS-TNTE*, it is possible that with more repetitions or another effector, such as *UAS-Kir2.1* or *UAS-Hid*, we may uncover a role of *ppk23*$^{+}$ GRNs for male *and* female detection in FIFIMMA. It is worth noting that the expression of *UAS-TNT* in *Gr5a-GAL4* does not reduce aggression in the presence of dead females, suggesting that the *ppk25* silencing experiments are unlikely to be due to *UAS-TNT*’s nonspecific effects on aggression.

From the above experiments, it is difficult to tell whether male or female-sensing GRNs are necessary for aggression, since both female and male CHs are present in the female presentation assay. Thus, in order to test whether the silencing results of *ppk25*$^{+}$ GRNs (and trends seen in *ppk23*$^{+}$ GRNs) were due to a defect in male or female CH silencing, I silenced these GRNs in an assay without female CHs, by testing these flies in the presence of food. When I expressed *UAS-*

TNT, I was able to see a reduction in aggression by the Dickson group's *ppk23*$^{+}$ GRNs (Figure 5b, left). Nevertheless, since the control genotype *UAS-TNT* flies also fought less than the Dickson *ppk23-GAL4/IMPA* flies, it is not possible to tell whether the reduction in aggression was due to silencing of *ppk23*$^{+}$ GRNs, or due to nonspecific effects of *UAS-TNT*. The Scott group's *ppk23-GAL4* and Pikielny *ppk25-GAL4* showed trends, but did not show significance. When I used *UAS-Shibire*^{ts} to silence these GRNs, I found that the Scott *ppk23-GAL4* and Pikielny *ppk25-GAL4* showed a reduction in aggression (Figure 5b, right).

These results were seemingly unexpected, given that *ppk25*$^{+}$ GRNs should only detect female CHs (Starostina et al., 2012; Vijayan et al., 2014), and there is no female CH in the arena when male flies are presented with food as the only appetitive resource. These results may be due to either technical reasons or biological reasons. Technical reasons could include that *ppk25*$^{+}$ GRNs also label male CH-sensing GRNs, or that the expression of neuronal inhibitors led to a nonspecific behavioral suppression. Biological reasons could include that silencing *ppk25*$^{+}$ GRNs affects aggression independent of female CH detection by unknown mechanism, or that *ppk25*$^{+}$ GRNs are involved in detection of cues present either in the food or in males, which were not considered in previous studies. Further studies are needed to distinguish from these possibilities in order to identify the precise mechanism by which *ppk25*$^{+}$ GRNs are required for male-male aggression.

ppk23^{+} GRNs are sufficient for increase in aggression

Although I was never able to figure out the reason for these seemingly paradoxical results of the *ppk25*$^{+}$ GRN silencing experiments, I sought to test whether activation experiments by activation of *ppk25*$^{+}$ GRNs or *ppk23*$^{+}$ GRNs would give clarity. By testing male flies whose *ppk23*$^{+}$ or *ppk25*$^{+}$ GRNs are activated against live opponent males lacking male pheromones and/or in the presence of dead females lacking pheromones, it may be possible to test whether these GRNs play a role in male detection or female detection, or both.

To activate *ppk23*$^{+}$ and *ppk25*$^{+}$ GRNs, I went through a battery of available neuronal activators: *UAS-NaChBac* (a bacterial voltage-gated Sodium channel [Nitabach et al., 2006]), *UAS-NaChBac/tubulin-Gal80ts* (a temperature-sensitive version of GAL80, used to restrict expression of *UAS-NaChBac* to adult flies [McGuire et al., 2003]), *UAS-dTrpA1* (temperature-sensitive Calcium channel [Rosenzweig et al., 2005]), *UAS-ChR2* (channelrhodopsin-2, a light-sensitive microbial opsin [Boyden et al., 2005; Suh et al., 2007]), and *UAS-ReACh* (red-shifted ChR2 [Inagaki et al., 2013]). I used these effectors to activate *ppk23*$^{+}$ and *ppk25*$^{+}$ GRNs in multiple contexts: 1) against oe- opponent males, 2) against oe- opponent in the presence of dead oe- female, 3) against same genotype opponent in the presence of food, 4) against same genotype opponent in the presence of dead oe- female. When paired with oe- opponent males (conditions 1 and 2), no condition gave an obvious increase, if any, in male-male aggression (data not shown). In the presence of food (condition 3), both *ppk25*$^{+}$ and *ppk23*$^{+}$ activation

by *UAS-NaChBac* increased aggression when the male flies were paired in isogenic pairs (data not shown). In addition to *NaChBac/Gal80ts*, I was also able to see an increase in aggression by using *UAS-ReACh* with *ppk23-GAL4*, but not *ppk25-GAL4* (data not shown, ppk23: more than half of >12 pairs tested, ppk25: none of the >12 pairs tested). In particular, *ppk23*$^{+}$ GRN activation gave male-male courtship behavior when the light was on, followed by male-male aggression after the light was turned off. Although these results suggested that the activity in *ppk23*$^{+}$ and *ppk25*$^{+}$ GRNs is sufficient to increase male-male aggression, the context tested did not allow us to test whether activation of these GRNs can bypass the requirement for male or female pheromones. Finally, I tested whether the activation of *ppk23*$^{+}$ and *ppk25*$^{+}$ GRNs can bypass the requirement for female pheromones by presenting isogenic pairs in the presence of an oe- female resource (condition 4). Using isogenic pairs with *ppk25-GAL4* and *ppk23-GAL4* activated by *UAS-NaChBac/tub-Gal80ts*, male flies increased aggression in the presence of an oe- female. (Figures 6a and 6b). These results demonstrate that in the absence of female-specific CHs, *ppk23*$^{+}$ and *ppk25*$^{+}$ GRN activation increases aggression, bypassing the requirement for female CHs.

These experiments are in apparent contradiction to the proposed idea that *ppk25*$^{+}$ GRNs are activated by female CHs, which in turn cause courtship behavior in males for the following reasons: 1) activation of *ppk25*$^{+}$ GRNs by *UAS-ReACh* did not increase courtship, while activation of *ppk23*$^{+}$ GRNs increased courtship and 2) silencing *ppk25*$^{+}$ GRNs in the presence of food where there is no female

pheromone decreased male-male aggression. These results could be explained if *ppk25*$^{+}$ GRNs either do not label female-sensing GRNs or enough female-sensing GRNs to give an activation phenotype but label some male-sensing GRNs, apparently enough to give both activation (increased aggression) and silencing results (decreased aggression in the presence of food without females). It is important to note that no study has thus far reported neuronal activation of *ppk25*$^{+}$ GRNs. Thus, it is unclear whether these results suggest a technical failure to activate *ppk25*$^{+}$ GRNs to induce courtship in males, or that the proposed hypothesis that activation of *ppk25*$^{+}$ GRNs leads to courtship should be revised. Nevertheless, at minimum, these results suggest that *ppk23*$^{+}$ and *ppk25*$^{+}$ GRNs play a role in male-male aggression in some contexts.

Binary chemosensory code model for male-male aggression

If 7,11-HD only promotes courtship toward females in single males, then how does it also promote aggression in the presence of another competing male? Similarly, food cues only promote feeding in single flies, but in the presence of another competing male, it also promotes aggression. These data suggest a possibility that the aggression requires not only the presence of resource, such as females or food, but also the presence of male-specific CHs; that is, only the detection of both male and female CHs can increase aggression. Although this was a particularly appealing hypothesis, previous experiments only tested the requirement of male-specific CHs in the context of food competition (Wang et al.,

2011), but not in the context of FIFIMMA. Thus, I sought to test whether male pheromones were required in FIFIMMA, which would suggest that male and female pheromones depend on one another to increase male-male aggression.

To test this, I paired wild-type male flies with target oe- or oe+ males, as described previously (Wang et al., 2011), and presented these pairs with oe- or oe+ females. When I tested these four conditions, I found a binary logic for male-male aggression in the presence of females (Figure 7a for aggression and 7b for courtship)**:** a) When a wild-type male fly was paired with another fly without any gustatory cues (oe- male), this led to increased MMC, as it was previously shown (Billeter et al., 2009; Fernández et al., 2010; Thistle et al., 2012; Wang et al., 2011). b) In the presence of male CHs (oe+ males), but without any appetitive resource cues, wild-type males suppressed courtship, but did not increase aggression. c) In the presence of only appetitive gustatory cues from females (oe+ female) but not male-specific cues (oe- male), wild-type males increased courtship toward females, but did not increase aggression. d) Finally, in the presence of both male CHs and female CHs, wild-type male flies fought. These data suggest that detection of male cues and appetitive cues from females constitute a logical “AND” gate for aggression. Since the receptor neurons for male and female CHs are known, these data suggested that integration of two distinct classes (male and female) of gustatory cues leads to male-male aggression.

ppk23^{+} GRNs and *ppk25*$^{+}$ GRNs - constructs

Dissecting the respective functions of male CH-sensing GRNs and female CH-sensing GRNs in FIFIMMA would demonstrate that the binary "logic gate" indeed functions as proposed. Nevertheless, this required the development of new genetic tools in order to selectively manipulate male CH-sensing GRNs independently of female CH-sensing GRNs. This was not possible using currently-available tools, since *ppk23-GAL4* labels both male and female CH-sensing GRNs (Thistle et al., 2012), while *ppk25-GAL4* only labels putative female CH-sensing GRNs (Vijayan et al., 2014), precluding selective manipulation of male CH-sensing GRNs. Furthermore, the confusing results with *ppk23*$^{+}$ and *ppk25*$^{+}$ GRN silencing experiments could, in part, be resolved by the development of new genetic tools, which could be used to manipulate male and female CH-sensing GRNs separately.

Since *ppk25*$^{+}$ GRNs are thought to be specific to female pheromone sensing according to some reports (Vijayan et al., 2014), it remained a possibility that *ppk25* promoter may be a useful tool to subdivide *ppk23*$^{+}$ GRNs. By using the GAL4/UAS system in combination with the LexA/LexAop or GAL80 system in *ppk23*$^{+}$ and *ppk25*$^{+}$ GRNs, it would be possible to dissect these overlapping populations of neurons functionally. Therefore, I sought to make *ppk25-GAL80* and *ppk25-LexA* transgenic flies, which can then be used to selectively manipulate these two classes of GRNs: ppk23(+)ppk25(-) male-sensing GRNs and ppk23(+)ppk25(+) female-sensing GRNs (schematic drawing in Figure 7d).

Although the Pikielny lab sent us their *ppk25-LexA* transgenic flies, I could

not verify the expression when it was used to drive a reporter (*LexAop-GFP*). A possible reason for this was because this version of *ppk25-LexA* used an older version of *GAD* (GAL4 activation domain), which does not efficiently drive the expression of reporters in the fly (Pfeiffer et al., 2010). Thus, I sought to make my own version of the *ppk25-LexA*, with a human p65 activation domain and a nuclear localization signal (NLS), both of which enhance LexA-driven expression compared to older versions of LexA with GAD and no NLS (Pfeiffer et al., 2010). In addition to the *ppk25-LexA*, I also used the same approach to generate *ppk25-GAL80*, which could be used to block expression in *ppk25*$^{+}$ GRNs. The original *ppk25-GAL4* line from the Pikielny lab was designed by cloning both the 5'UTR and 3'UTR of the *ppk25* locus, both of which were used to drive the expression of the GAL4 protein (Starostina et al., 2012) (Figure 8a). The expression pattern of *ppk25-GAL4* using this approach revealed that *ppk25-GAL4* was expressed in putative female pheromone receptor GRNs (Starostina et al., 2012), which overlapped with *fruitless* but not *CheB42a*, which has been shown to be present in support cells surrounding gustatory receptor neurons (Ben-Shahar et al., 2010; Bray, 2007; Lin et al., 2005).

With the transgenic flies from Genetic Services, I used double reporters with nuclear localization signals (*UAS-nlsTdTomato/LexAop-nlsGFP*) to analyze the expression pattern of the newly generated *ppk25-LexA* and *ppk25-GAL80* flies. These experiments revealed that the *ppk25-LexA* was expressed in most *ppk25*$^{+}$ cells in the male foreleg (Figure 8b). From the most distal tarsal segments,

ppk25-LexA was expressed in 2-3 cells, while *ppk25-GAL4* was present in 2-3 cells in TA5 (100% overlap). In TA4, *ppk25-LexA* was present in 6-8 cells out of 7-8 cells (75 to 100% overlap). In TA3, *ppk25-LexA* was present in 5-7 cells out of 6-7 cells in TA3 (100% overlap). These numbers show that the expression of pattern of *ppk25-LexA* faithfully recapitulates the *ppk25-GAL4* expression pattern, and the number of neurons being labeled are similar, as previously reported (Starostina et al., 2012). No expression was detected in the brain, unlike *ppk25-GAL4*, which is weakly expressed in the antennal lobe (data not shown, also reported in [Starostina et al., 2012]). These numbers are in agreement with the previously-reported expression pattern of ppk25-GAL4 (Liu et al., 2012).

Next, to check whether *ppk25-LexA* represents a subset of *ppk23*$^{+}$ GRNs, as was predicted by behavioral and physiological data from other groups (Thistle et al., 2012; Vijayan et al., 2014), I co-expressed *ppk25-LexA* along with *ppk23-GAL4* (Figure 8c). Since only the Dickson lab's *ppk23-GAL4* showed a gain-of-function phenotype with both *UAS-NaChBac/tub-Gal80ts* and *UAS-ReACh*, I used this version of *ppk23-GAL4*. As was predicted, all of *ppk25*$^{+}$ GRNs represented a subset of *ppk23*$^{+}$ GRNs, as all *ppk25*$^{+}$ GRNs observed were also *ppk23*$^{+}$ (Figure 8c and Figure 8d, note: some green-colored epi-fluorescence from cuticle of is not true expression). In the most distal segment TA5 in the foreleg, ~20% of *ppk23*$^{+}$ GRNs were labeled by *ppk25-LexA* (2-3 out of 10), ~50% in TA4 (6-8 out of 14-16), and ~50% in TA3 (5-7 out of 14). These results

are summarized in the Table (Figure 8e).

Previously, it was suggested that *ppk23*$^{+}$ GRNs occur in pairs, one sensitive to male CHs, and another sensitive to female CHs (Thistle et al., 2012). This was true in most cases, as ppk25(+)ppk23(+) cells ("F cells") were almost always accompanied by adjacent ppk25(–)ppk23(+) cells ("M cells," which had similar axonal projections (Figure 8d, Figure 8e). In some cases, there were unpaired *ppk23*$^{+}$ cells, which did not have adjacent F cells in pairing (28 paired out of 40 in TA3-5, ~70%). This could reflect either insufficient labeling of F cells by *ppk25-GAL4* and *ppk25-LexA*, or nonspecific labeling by *ppk23-GAL4*. The latter is more likely, as *ppk23-GAL4* was reported to have broader expression than *fru-GAL4*, which were found in ~75% of *ppk23*$^{+}$ cells (Toda et al., 2012). Thus, it is likely that 70 to 75% of *ppk23*$^{+}$ GRNs are male and female sensing GRNs, half of which are *ppk25*$^{+}$ GRNs (Table in Figure 8e).

Finally, I checked whether *ppk25-GAL80* could suppress the expression of *ppk25-GAL4* and a subset of *ppk23-GAL4*. This analysis revealed that *ppk25-GAL80* completely suppressed the expression of *ppk25-GAL4* driver, as no expression was detected in any cells in the brain or in the leg (Figure 9a). Co-expression of *ppk25-GAL80* with *ppk23-GAL4* revealed that GAL80 inhibited expression of the reporter in a small number of cells, as would be expected by the number of *ppk25-GAL4* expressing GRNs (Figure 9b). Taken together, these results suggest that *ppk25-GAL80* works as expected.

Preliminary behavioral experiments using these new flies revealed that *ppk25-LexA*, like the *ppk25-GAL4*, do not increase courtship upon activation by *LexAop-ReACh* or *UAS-Chrimson*. *ppk23-GAL4/ppk25-GAL80* did not appear to fight, although more experiments are needed to substantiate this result. Although further behavioral and Calcium-imaging experiments are necessary to fully characterize the functional role of *ppk25-GAL4* or *ppk25-LexA* expressing GRNs, these tools will serve to test many hypotheses.

Conclusion

In nature, upon encountering conspecifics, males must correctly determine the gender, and act accordingly. In many species, females are both a target of courtship as well as an appetitive resource to fight over. Upon detection of a female, males must decide whether to pursue the female first, or fight with other competing males; this decision should, at minimum, depend on the presence of another male. Identification of sensory mechanisms, which mediate this behavioral decision between courtship and aggression, is a necessary first step toward understanding how the brain makes decisions by integrating multiple sensory inputs.

In *Drosophila*, males are known to compete over females in the presence of food, but it is unclear whether females alone can increase aggression (Billeter et al., 2009; Chen et al., 2002; Fernández et al., 2010; Fernández and Kravitz, 2013; Hoffmann and Cacoyianni, 1990; Lacaille et al., 2007; Skrzipek et al.,

1979; Wang and Anderson, 2010; Wang et al., 2011; Yuan et al., 2014). I show that females can increase male-male aggression in the absence of food. Unlike single-housed males, who show a high level of aggression over food, group-housed males do not show much aggression in the presence of food. In contrast, in the presence of females without food, group-housed males show high levels of aggression. Thus, females by themselves can robustly increase male-male aggression. Since males are attracted to females, it is possible that the effect of females to increase aggression is correlated with increases in inter-male proximity and male-male encounter duration. Nevertheless, I show that the presence of females does not change the male-male encounter duration. Furthermore, distributing 10 females evenly throughout the arena could still increase male-male aggression, suggesting that the food-independent, female-induced male-male aggression (FIFIMMA) is not dependent on attraction to a single physical location. In addition to male-male aggression, I found that females also increased locomotion and courtship, suggesting that females increase either general or social arousal. Increases in locomotion cannot fully account for the increase in aggression, as normalization of aggression by locomotion still showed a robust difference. Taken together, these data suggest that females increase male-male aggression, and that it is independent of effects on proximity or locomotion.

Previous studies have identified mechanisms by which male flies detect the presence of females via female-specific CH pheromones (Antony and Jallon,

1982; Ferveur, 2005; Jallon, 1984; Lu et al., 2012; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012). I showed that female CH pheromones are necessary to increase FIFIMMA, as female flies without female pheromones either by washing with solvent or by genetic ablation of CH-producing oenocytes (oe- females) decreases male-male aggression. In addition, I showed that female perfumed CHs are sufficient to increase FIFIMMA, and that 7,11-HD perfumed on either oe- males or oe- females is sufficient to mimic the effect of FIFIMMA, suggesting that the 7,11-HD can increase aggression without any other female-specific cues. 7,11-HD presented alone on filter paper did not increase FIFIMMA, suggesting that there may be signals present on male and female fly bodies, which must be present in order for 7,11-HD to promote FIFIMMA. Taken together, these data suggest that female CHs and particularly 7,11-HD, presented together with any fly body, can promote male-male aggression.

Recent studies have shown that male flies detect sex-specific pheromones using pheromone-sensing GRNs as well as ORNs (Lu et al., 2014; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; van der Goes van Naters and Carlson, 2007; Vijayan et al., 2014). In particular, *ppk23*$^{+}$ GRNs respond to male and female CHs (Thistle et al., 2012; Toda et al., 2012), while *ppk25*$^{+}$ GRNs respond to female CHs (Vijayan et al., 2014), suggesting that these may mediate female CH detection in FIFIMMA. By silencing *ppk25*$^{+}$ GRNs using tetanus toxin light chain (*UAS-TNT*) and a dominant-negative mutant dynamin (*UAS-Shibire*^{ts}), I showed that *ppk25*$^{+}$ GRNs are necessary for FIFIMMA. Although silencing *ppk23*$^{+}$

GRNs did not show a statistically significant decrease in FIFIMMA, strong trends were observed, suggesting that FIFIMMA may depend on both *ppk23*$^{+}$ and *ppk25*$^{+}$ GRNs. By activating *ppk23*$^{+}$ and *ppk25*$^{+}$ GRNs by expressing voltage-gated Sodium channel (*UAS-NaChBac/tubulin-Gal80ts*) in adults, I showed that the activity in these GRNs is sufficient to increase aggression in the presence of oe- females, suggesting that they can bypass the requirement for female CHs in FIFIMMA.

Although more experiments are necessary to confirm these results with *ppk23*$^{+}$ and *ppk25*$^{+}$ GRNs, it is important to note that in the presence of food and without any female CHs, silencing the *ppk23*$^{+}$ and *ppk25*$^{+}$ GRNs decreased male-male aggression. This result is difficult to interpret, since *ppk23*$^{+}$ and *ppk25*$^{+}$ GRNs have been implicated only for female-CH detection; thus, silencing them should have no effect on food-induced male-male aggression. There are several possibilities, which may explain this apparently paradoxical result and experiments to test these possibilities:

1) *ppk25*-silencing experiments show nonspecific decrease in behavior, and thus decrease in aggression is a confound.

Given that relatively few repetitions of *ppk23*$^{+}$ and *ppk25*$^{+}$ GRN silencing experiments were performed and there were few or no experiments performed with control genotypes (*UAS-TNT/+* and *UAS-Shibire/+*), more experiments are necessary to demonstrate that the effect of *ppk25*-silencing is statistically significant. If *ppk25*$^{+}$ GRN silencing does not show a statistically significant

decrease compared to control genotypes, then we cannot conclude that *ppk25*$^{+}$ silencing decreases aggression on food. Furthermore, we should test whether ppk25-silencing decreases aggression with other neuronal silencers, such as *UAS-Hid* or *UAS-Kir2.1*. By performing these additional experiments, it should be possible to show whether or not *ppk25*$^{+}$ GRNs are truly necessary for male-male aggression on food.

2) *ppk25-GAL4* expression is not specific to female CH-responsive cells, and it is expressed in some male CH-responsive GRNs.

Given that no study has shown that activation of *ppk25*$^{+}$ GRNs leads to courtship, and that our results show that activation of *ppk25*$^{+}$ GRNs by *UAS-ReACh* or *UAS-Chrimson* does not increase courtship while the same manipulations with *ppk23*$^{+}$ GRNs does, it is possible that *ppk25*$^{+}$ GRNs may not specifically respond to female CHs. Indeed, previous studies examining *ppk25*$^{+}$ GRNs did not examine the response of these GRNs in *ppk25*$^{+}$ GRNs, but only in some bristles in the forelegs (Vijayan et al., 2014).

Silencing male CH-sensing GRNs should lead to both decrease in male-male aggression and increase in male-male courtship. Thus, in order to test whether *ppk25*$^{+}$ GRNs also label male-sensing GRNs, we can repeat the silencing experiments with *ppk25*$^{+}$ GRNs and observe whether this results in a change in male-male courtship. Decrease in male-male courtship depends on detection of 7-T via *ppk23*$^{+}$ GRNs; thus, if *ppk25*-silencing increases male-male courtship, it is likely that the effect of *ppk25*-silencing on food-induced male-male aggression is

due to the detection of male-CH-sensing GRNs. If *ppk25*-silencing does not affect male-male courtship, we can conclude that *ppk25*$^{+}$ GRNs are not affecting male CH detection, and thus *ppk25*$^{+}$ GRNs are likely specific to female CHs.

3) Weak expression of *ppk25-GAL4* seen in the brain and olfactory neurons is responsible for decrease in food-induced male-male aggression.

ppk25-GAL4, unlike *ppk25-LexA*, is expressed in two subsets of *fru*$^{+}$ ORNs (Starostina et al., 2012), which respond to fly odors (van der Goes van Naters and Carlson, 2007). Thus, it is possible that the reduction in male-male aggression is due to silencing these ORNs. To test this possibility, we can use *ppk25-LexA*, which does not show any expression in ORNs. If silencing *ppk25-LexA* does not show a decrease in food-induced aggression but decreases female-induced aggression, then we can conclude that *ppk25-GAL4* has some non-specific expression most likely in ORNs or CNS that is responsible for decreasing male-male aggression.

4) *ppk25-GAL4* is specific to female CH-responsive cells but *ppk25*$^{+}$ GRNs have female-CH-independent basal activity, which is necessary for male-male aggression. Or, *ppk25*$^{+}$ GRNs respond to some generic pheromone that is present on both females and males.

To test this possibility, we can perform Calcium-imaging experiments to test whether *ppk25*$^{+}$ GRNs show high levels of basal activity, and see whether silencing *ppk25*$^{+}$ GRNs leads to decrease in this basal activity. Furthermore, we can also image the response of other neurons such as *ppk23*$^{+}$ GRNs when *ppk25*$^{+}$

GRNs are silenced to test whether silencing *ppk25*$^{+}$ GRNs leads to a decrease in the activity of male CH-sensitivity. Finally, we can also test whether *ppk25*$^{+}$ GRNs respond to any generic fly pheromones, which are present in both males and females.

The above experiments will shed light on the question of why *ppk25*$^{+}$ GRN silencing decreases aggression in the absence of any female CH cues. Once this question is answered in a satisfactory manner, and assuming that *ppk25-LexA* is specific to female-sensing GRNs, we can continue to test the predictions of the binary chemosensory code at the level of single GRNs (Figure 7c). Thus far, previous studies (Fernández et al., 2010; Thistle et al., 2012; Wang et al., 2011) and my results have demonstrated the first row (no male or female cues or *ppk23*$^{+}$ silencing = male-male courtship, no aggression) and the fourth row (male cues + female cues or *ppk23*$^{+}$*/ppk25*$^{+}$ activation = aggression and courtship). By using the newly generated tools, we can test the remaining second and third rows. To test the second row (male cues only or 7-T sensing GRN = decreased male-male courtship and no aggression), we can activate 7-T sensing GRNs by using *ppk23-GAL4/ppk25-GAL80* and test whether this leads to just reduction of male-male courtship but no aggression. To test the third row, we can use *ppk25-GAL4* or *ppk25-LexA* to test whether we can increase courtship without any aggression. Although *ppk25-GAL4* activation by *UAS-ReACh* or *UAS-Chrimson* did not result in any increase in courtship behavior, this could be due to the same confounds, which led to confusing silencing results. Furthermore, other experimental contexts

could be explored, such as having live oe- females with oe- males in the arena to test whether activation of *ppk25-GAL4* or *ppk25-LexA* leads to increased courtship. Ultimately, these results indicate that male flies' choice between aggression and courtship depends on two sex-specific gustatory pheromones, which are detected by *ppk23*$^{+}$ GRNs. These tools may be explored in future studies to dissect how these two sensory pathways (male- and female-sensing) converge in the central nervous system to give rise to the behavioral decision to increase aggression.

Materials and Methods

Behavioral Assays and Analysis

Behavioral assays were performed as described in Chapter 2. For the most part, group-housed flies were used for experiments in Chapter 3. All experiments were performed in a 40 mm x 50 mm behavior chamber previously described (Hoyer et al., 2008). All presentations of dead flies involved group-housing virgin females or males for 3-7 days and then freeze-killing them in -20ºC freezer for 30 minutes. The freeze-killed flies were carefully laid on top of 1% agarose on their sides and 1% agarose was used to cover their genitals, legs and wings to expose their abdomen, where cuticular hydrocarbon producing oenocytes reside. Experiments with *UAS-ReACh* and *UAS-Chrimson* were used as described previously (Inagaki et al., 2013), where 12-well chambers were used, with each arena occupied by a pair of male flies. All other conditions were identical to the conditions described in

Chapter 2.

Manipulations of cuticular hydrocarbons

For hexane washing experiments, the same protocol was used as described previously (Savarit et al., 1999). Briefly, 50 µL of hexane was used to wash single flies. To perfume hydrocarbons, two different methods were used. First, to transfer CH pheromones from live flies onto oe- flies, I adapted the procedure previously described (Wang et al., 2011), where white-eyed donor flies were housed together with oe- flies overnight in small vials. In order to perfume synthetic pheromone, 7,11-HD and 7,11-ND, a similar protocol was adapted from the same study (Wang et al., 2011). Briefly, 1.0 µL of synthetic 7,11-HD or control solvent hexane was placed onto a small cutout of filter paper. This filter paper was then placed in a 5-ml glass vial with 5-10 flies and vortexed twice for 15 seconds at slow speeds. The perfumed flies were then freeze-killed as described above.

Fly Stocks and Rearing Conditions.

All fly stocks were reared as described in Chapter 2. All transgenic flies were backcrossed for 6 generations into the CS background. Two strains of *ppk23-GAL4* flies used were gifts from Kristin Scott Lab and Barry Dickson Lab. *ppk25-GAL4* flies were a gift from the Claudio Pikielny Lab. *UAS-Shibire^{ts}* flies were flies were a gift from obtained from the Gerald Rubin Lab (Pfeiffer et al., 2012). *pJFRC107-13XLexAop2-IVS-nlsGFP* and *pJFRC106-13XLexAop2-IVS-nlstdTomato* flies were gifts from the Gerald Rubin Lab.

Generation of transgenes

New transgenic flies described (*ppk25-LexA* and *ppk25-GAL80*) were generated using plasmids from the Pikielny Lab, and pUC19 backbone as previously described (Pfeiffer et al., 2010). Starting with the ppk25-GAL4 plasmid from the Pikielny lab, I subcloned the *ppk25* 3'UTR fragment by first introducing *XbaI* restriction enzyme sites and ligating it with the *XbaI*-digested pBPGal80Uw-6 and pBPnlsLexA::p65Uw (described in [Pfeiffer et al., 2010]). Next, I subcloned the ppk25 5'UTR fragment from the ppk25-GAL4 plasmid into the PCR8 vector using the PCR8/TOPO kit. Then, I combined the ppk25 5'UTR inside the PCR8 vector with the pBPGal80Uw-6–ppk25 3'UTR and pBPnlsLexA::p65Uw—ppk25 3'UTR vectors using the GATEWAY system. After sequencing the final products, ppk25-LexA and ppk25-GAL80, to make sure that everything was done with correct orientation, these plasmids (see Figure 8a) were injected into the multiple genomic loci by Genetic Services.

Statistical Analyses

Statistical analyses were performed exactly as described in Chapter 2.

. p values are abbreviated using asterisks. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, N.S. (not significant): $p > 0.05$.

Figure 1

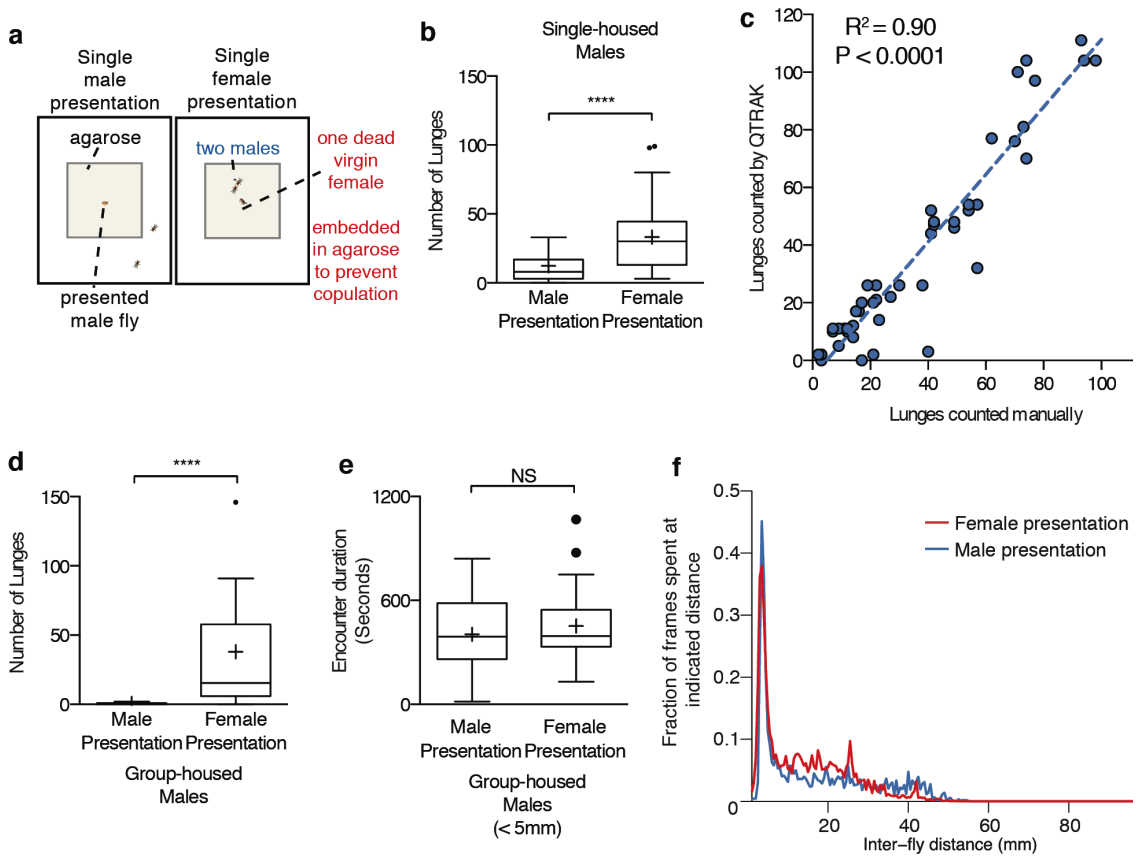
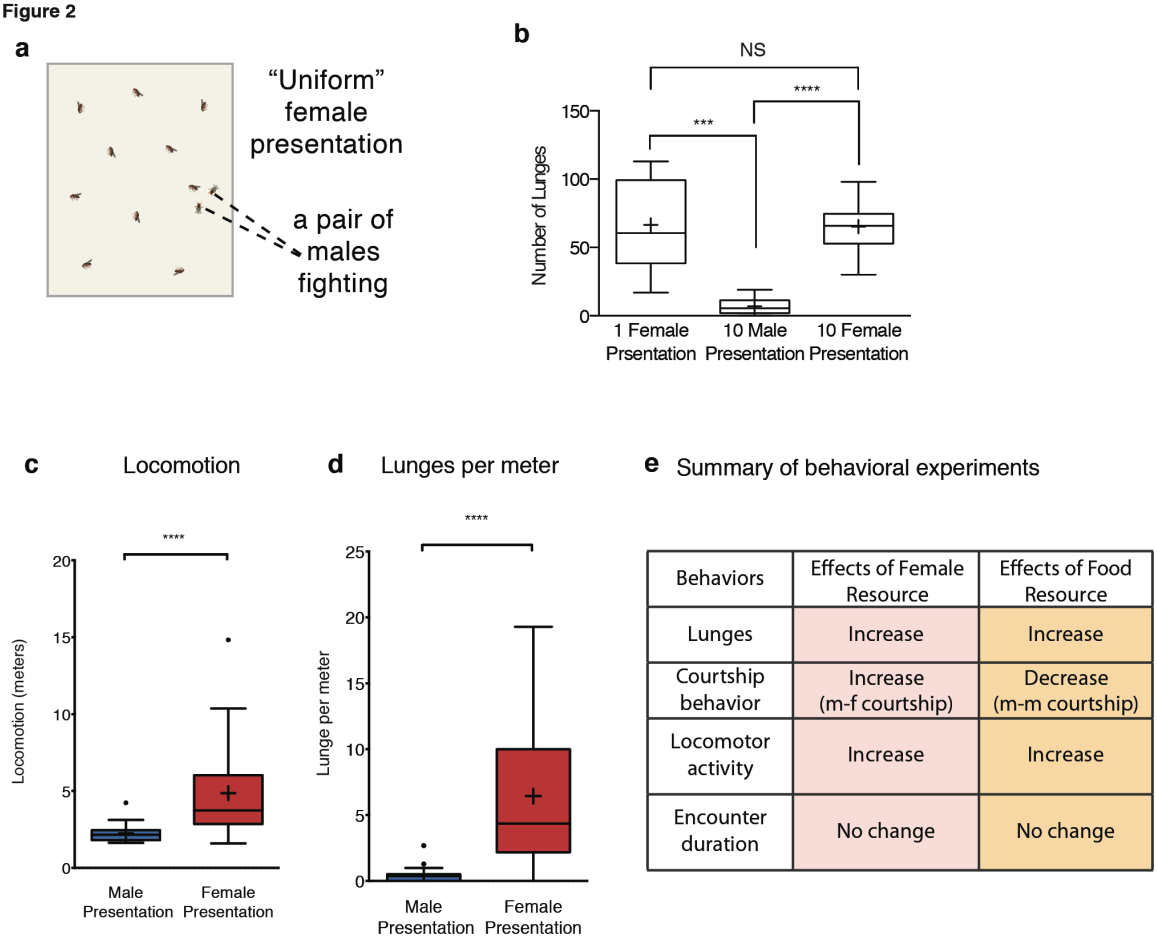


Figure 1. Females are sufficient to increase aggression.

(a) Schematic diagram of the aggression arena used for the experiments. The arena shown is a 40 x 50 mm arena. A freeze-killed female is partially embedded in 1% agarose to prevent copulation. A pair of male flies is illustrated at scale for comparison. Everything is in scale. (b) Single-housed flies performed more lunges during the observation period in the presence of a dead female vs. a dead male. n = 33, 53. (c) Comparison of manual scoring of number of lunges vs. analysis software scoring of number of lunges. n = 48. R^2 = 0.90. P < 0.0001. (d) Group-housed flies performed more lunges during the observation period in the presence of a dead female vs. a dead male. n = 30, 26. (e) Female presentation does not

increase encounter duration between males. Encounter duration is the sum of the amount of time male flies spent within 5 mm (1 – 2 body lengths of each other, depending on orientation) of each other. n = 30, 38. (f) Female presentation does not change the amount of time flies spent at various distances from each other. The inter-fly distance histogram shows the amount of time flies spend (y-axis) at a given distance from each other (x-axis). The trace is the median trace from 30 and 38 male-male pairs for male and female presentation, respectively.

Figure 2



Behaviors	Effects of Female Resource	Effects of Food Resource
Lunges	Increase	Increase
Courtship behavior	Increase (m-f courtship)	Decrease (m-m courtship)
Locomotor activity	Increase	Increase
Encounter duration	No change	No change

Figure 2. Females increase aggression independent of effects to increase proximity between male flies.

(a) Schematic diagram of the aggression arena used for the 10-female presentation assay. The arena shown is a 40 x 50 mm arena. 10 freeze-killed female are partially embedded in 1% agarose to prevent copulation. A pair of male flies is illustrated at scale for comparison. (b) Presentation of 10 females increases aggression compared to presentation of 10 males. Presentation of 10 females is indistinguishable from presentation of 1 female (n = 12, 12, 14 for 1 female, 10 male, and 10 females, respectively). (c) Presence of female increases locomotion.

(d) Presence of female increases male-male aggression when normalized by locomotion. (e) Presence of female increases general and social arousal, leading to an increase of multiple male behaviors, including aggression.

Figure 3

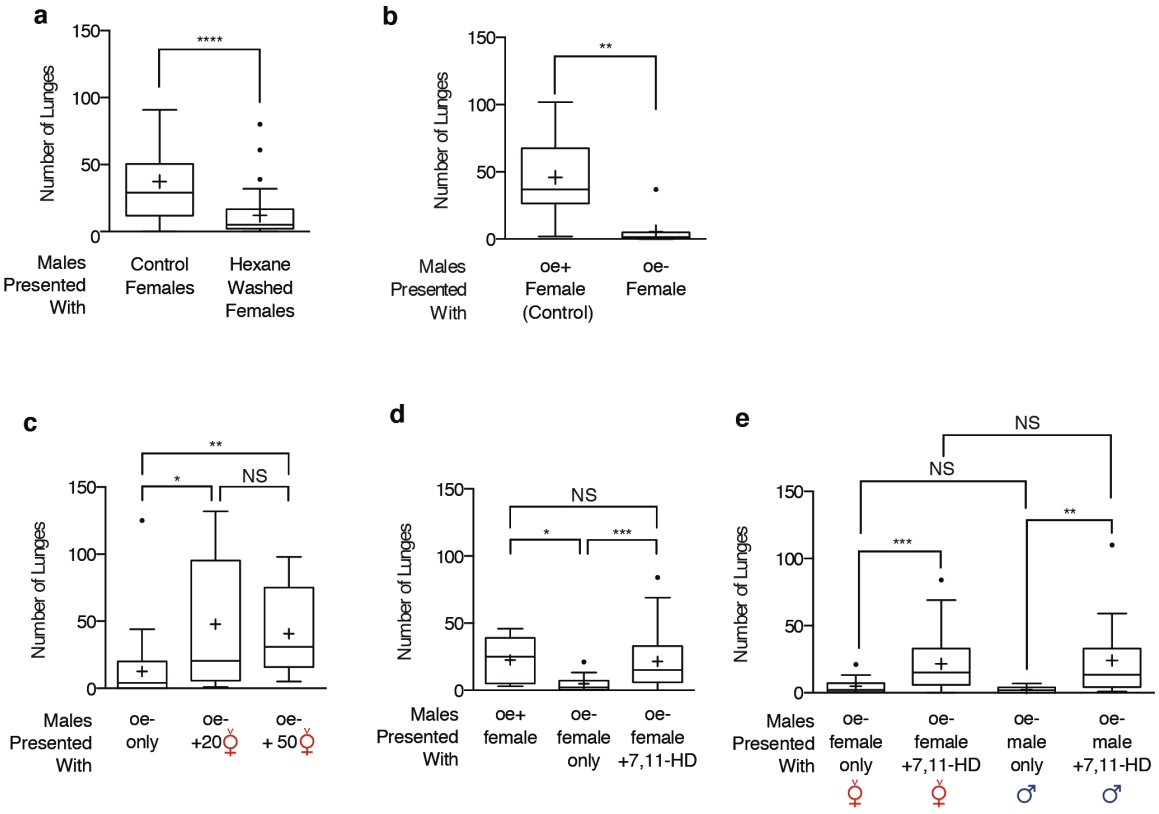


Figure 3. Female pheromones are necessary and sufficient to increase male-male aggression.

(a) Presentation of females washed with hexane reduces male-male aggression (n = 48, 36). (b) Presentation of oenocyte-ablated females without female pheromones (oe-) reduces aggression compared to control oe+ females with normal pheromone profile (n = 9, 10). (c) Presentation of oe- females perfumed with female pheromones restores female-induced male-male aggression. oe- females are housed by themselves or with white-eyed wild-type females in the vial overnight (n = 10, 13, 33). (d) Perfuming oe- females with 7,11-HD restores aggression. The level of aggression is indistinguishable from control oe+ females

(n = 5, 20, 45). (e) 7,11-HD is sufficient to increase aggression when perfumed on oe- males. The level of aggression is similar regardless of the sex of the perfumed fly (n = 20, 45, 12, 16). The oe-, oe- + 7,11-HD data are re-plotted from Figure 3d for comparison purposes.

Figure 4

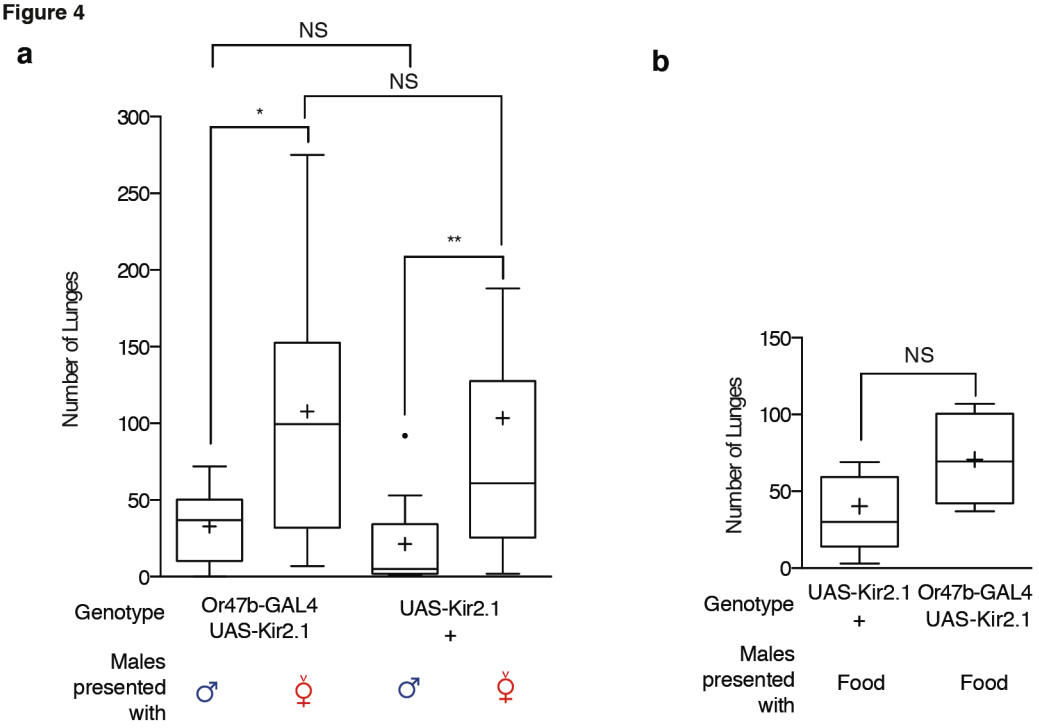


Figure 4. *Or47b*$^{+}$ olfactory receptor neurons (ORN) are not required for female-induced male-male aggression.

(a) In the presence of females, silencing *Or47b*$^{+}$ ORNs by expressing *UAS-Kir2.1* does not reduce aggression (n = 10, 26, 10, 33). (b) Silencing *Or47b*$^{+}$ ORN does not change the level of aggression in the presence of food (n = 4, 12).

Figure 5

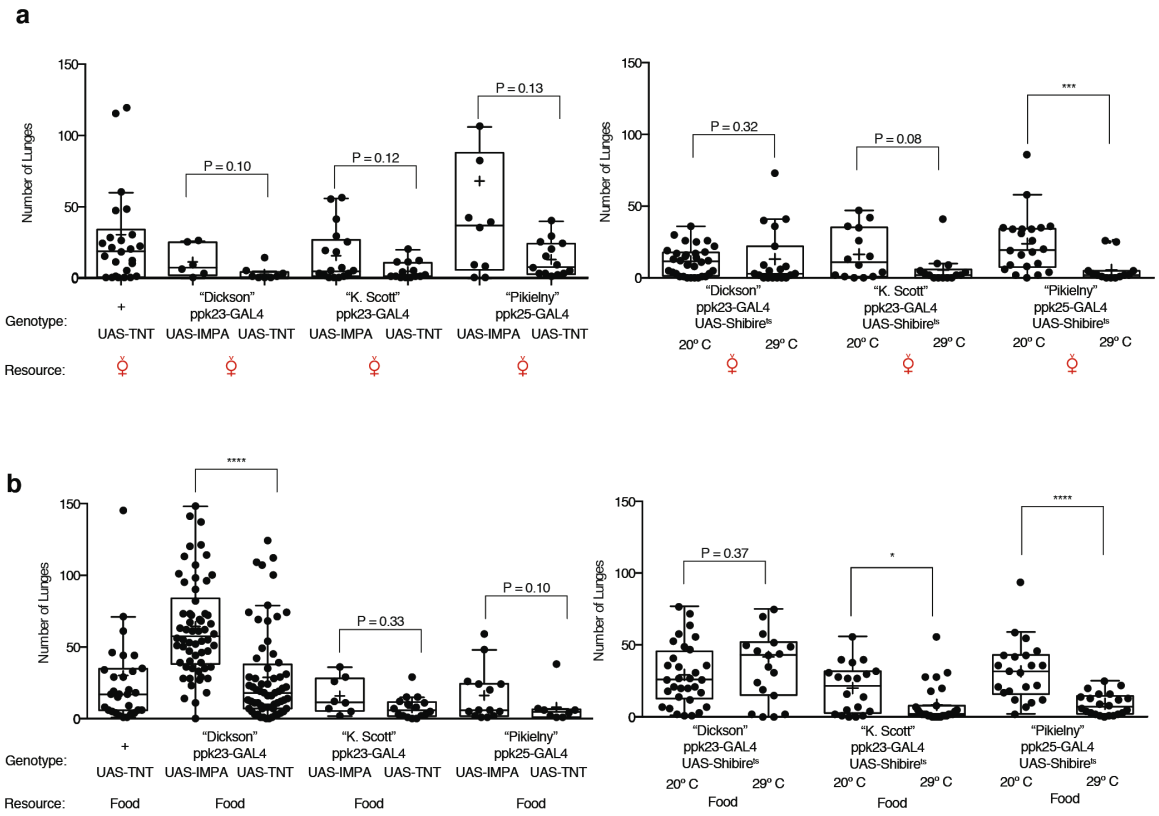


Figure 5. Preliminary data: *ppk25*$^{+}$ and *ppk23*$^{+}$ GRNs may be necessary for male-male aggression in some contexts.

(a) In the presence of females, silencing *ppk25*$^{+}$ GRNs reduces male-male aggression, while silencing *ppk23*$^{+}$ GRNs shows some trends. More replicates are necessary. The statistical comparisons are only between the paired brackets, without any additional multiple comparisons. Left: Silencing by expressing *UAS-TNT* or the control *UAS-IMPA* (n = 26, 6, 8, 17, 14, 10, 14). Right: Silencing by expressing *UAS-Shibire*^{ts} (n = 32, 19, 14, 14, 22, 13). (b) In the presence of food, *ppk23*$^{+}$ and *ppk25*$^{+}$ GRNs are necessary for male-male aggression. The statistical comparisons are only between the paired brackets, without any additional multiple

comparisons. Left: Silencing by expressing *UAS-TNT* or the control *UAS-IMPA* (n = 31, 62, 64, 14, 16, 8, 8). Right: Silencing by expressing *UAS-Shibire^{ts}* (n = 32, 19, 20, 28, 22, 22). Flies are single-housed for this assay, because group-housed flies do not show a high enough level of aggression.

Figure 6

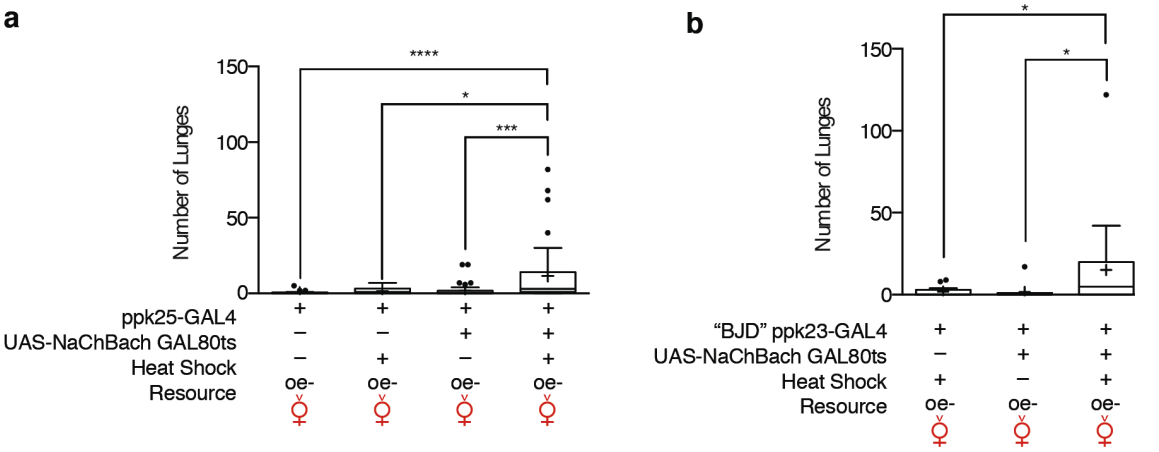
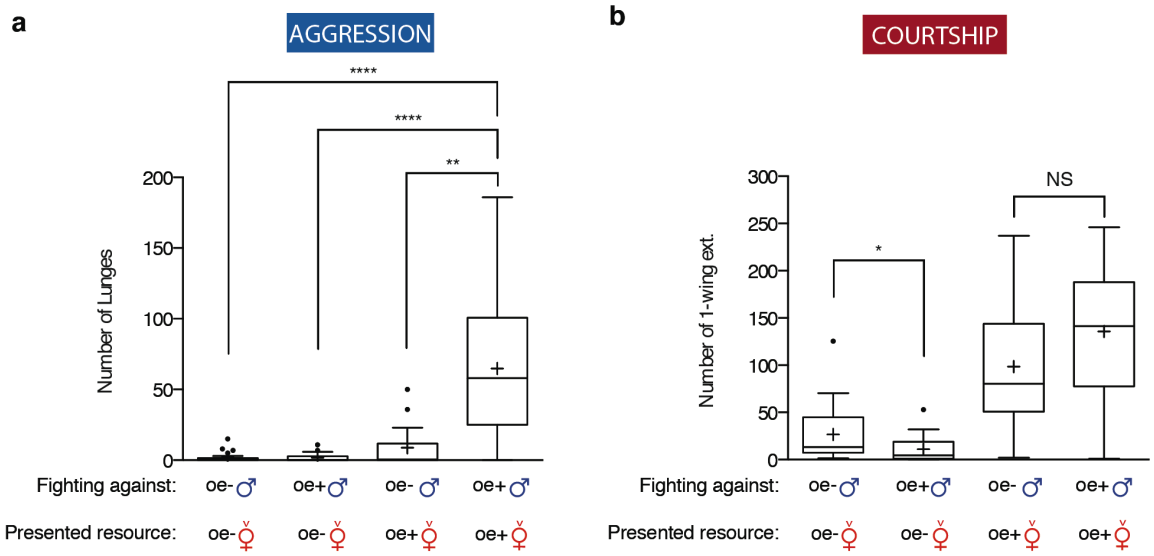


Figure 6. *ppk25*$^{+}$ and *ppk23*$^{+}$ GRNs are sufficient to increase male-male aggression in the absence of female or food.

(a) In the presence of oe- females, activation of *ppk25*$^{+}$ GRNs by expression of *UAS-NaChBac/tub-Gal80ts* increases male-male aggression in a genotype and heat-shock specific manner (n = 24, 31, 36, 39) (a) In the presence of oe- females, activation of *ppk23*$^{+}$ GRNs by expression of *UAS-NaChBac/tub-Gal80ts* increases male-male aggression in a genotype and heat-shock specific manner. (n = 16, 14, 23).

Figure 7



c **Proposed chemosensory code for male behavioral decision.**

ppk25⁻/ppk23⁺ GRN input (7-T, male detection)	ppk25⁺/ppk23⁺ or Gr5a⁺ GRN input (7,11HD or sucrose, resource detection)	Behavioral outcome
–	–	male-male courtship (MMC)
+	–	suppression of MMC
–	+	acquisition of resources
+	+	male-male **aggression** (competition over resource)

d

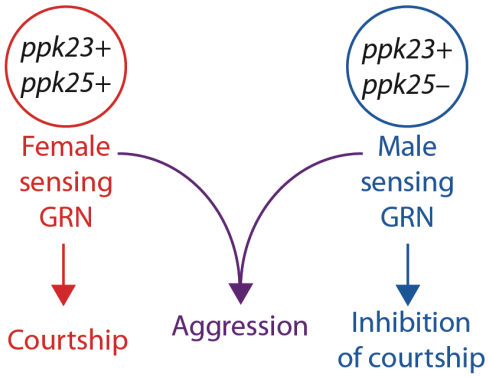
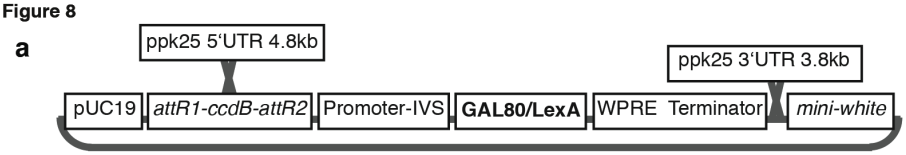
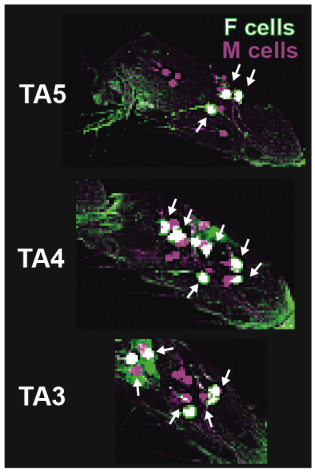


Figure 7. Female and male cues are both independently necessary for male-male aggression.

(a) In the absence of male and female cues, flies do not fight. When male cues are present but female cues are absent, flies do not fight. When female cues are present but male cues are absent, flies do not fight. In the presence of both male and female cues, flies fight (n = 22, 22, 14, 22). (b) Male cues suppress male-male courtship (manually scored), but male cues do not affect female pheromone-induced courtship (scored by CADABRA, n = 22, 22, 14, 22). (c) Proposed chemosensory code for male behavioral decision. Using only two inputs (male cues and female/food cues), it is possible to predict male behaviors between male-male courtship, suppression of male-male courtship, acquisition of resources and competition over resources. (d) Schematic diagram summarizing the binary chemosensory code.

Figure 8





e

	"M + F cells" ppk23-GAL4	"F cells" ppk25-GAL4	"F cells" ppk25-LexA(+) ppk23(+)	"M cells" ppk25-LexA(–) ppk23(+)
TA5	10	2	2-3	6-7
TA4	14-16	7-8	6-8	7
TA3	14	6	5-7	7

Figure 8. Construction of *ppk25*-related genetic reagents and testing (*LexA*)

(a) Schematic diagram showing the components that were used in making the

ppk25-LexA and *ppk25-GAL80* plasmids, which were used to make transgenic flies. (b) *ppk25-LexA* recapitulates *ppk25-GAL4* expression. *ppk25-LexA* was used in combination with *pJFRC107-13XLexAop2-IVS-nlsGFP* (abbreviated *nlsGFP*). *ppk25-GAL4* was used with *pJFRC106-13XLexAop2-IVS-nlstdTomato* (abbreviated *nlstdTomato*). Forelegs were mounted as a whole following a brief paraformaldehyde-fixing period, and subsequently imaged using a confocal microscope. Representative example of *ppk25-LexA/ppk25-GAL4* flies' forelegs is shown. (c) *ppk25-LexA* is expressed in a subset of *ppk23-GAL4* expressing GRNs. Representative example of *ppk25-LexA/ppk23-GAL4* flies' forelegs is shown. (d) Higher magnification of (c) showing overlap between *ppk23-GAL4* and *ppk25-LexA*. White arrows indicate where there are pairs of GRNs observed, one of which is white (*ppk23*(+)*/ppk25*(+)), which is putative female CH-sensing GRN ("F cell" and a magenta cell (*ppk23*(+)*/ppk25*(–)), which is putative male CH-sensing GRN ("M cell"). There are a few unpaired "M cells" that are not adjacent to any "F cells." (e) Table summarizing the number of GRNs counted in the forelegs (n > 5).

Figure 9

Figure 9. Testing of the ppk25-GAL80.

(a) *ppk25-GAL80* completely suppresses *ppk25-GAL4* expression. Representative example of *ppk25-LexA/ppk23-GAL4* flies' forelegs is shown. (b) *ppk23-GAL4* expression is partially suppressed by *ppk25-GAL80*. Left: *ppk23-GAL4* without GAL80. Right: *ppk23-GAL4* with *ppk25-GAL80*. There are several missing neurons

in both TA5 (more than 3) and TA4 (many). White arrows show the possible locations of missing *ppk25-GAL80*-expressing cells, which are likely missing due to the *ppk25-GAL80* activity.

A p p e n d i x

QUANTIATIVE MODELS OF RESOURCE-CONTROL OF FLY AGGRESSION

Inverted-U shape of aggression

In the preceding chapters, I described how two different resources, food and females, control male-male aggression through independent chemosensory mechanisms. Besides the differences in the sensory mechanisms processing these different sensory cues, there was also an apparent difference in dose-dependent response to food vs. females. In the case of food, there was a clear biphasic, dose-dependent increase and decrease in aggression, while in the case of females, one female seemed to robustly increase aggression as much as ten females*.

This dose-dependent way in which food promotes aggression, particularly the "inverted-U" response that we observe with food in *Drosophila*, is predicted by theoretical models and observed in a few other organisms (Carpenter and Macmillen, 1976) (reviewed [Maher and Lott, 2000; Peiman and Robinson, 2010]). Interestingly, this "inverse-U" shape is also seen in humans, where economic participation, which may be a form of competition in humans, rises and falls as the country's GDP per capita increases over time (Lopez-Feldman et al., 2011; Manyika et al., 2012). These observations suggest that strategic competition may be universal.

Most of these studies are observational rather than experimental, and there is a general paucity of data; for instance, the study by Carpenter *et al*. relied on 10 individuals and curve-fitting *by eye* (Carpenter and Macmillen, 1976). Furthermore, while many studies have observed a decrease in aggression as the

resource increases (Archer, 2009a; Hansen, 1986; Johnson et al., 2004) and others have observed the initial onset of aggression as food increases (Keenleyside and Yamamoto, 1962; Newman, 1956), very few studies have observed the complete inverse-U shape (Grant et al., 2002; Toobaie and Grant, 2013; Wyman and Hotaling, 1988).

In the fly, we observed a dose-response relationship between food and aggression. Furthermore, through careful manipulations of the amount of food, we were able to deduce that fly aggression shows an inverse-U shape response to changing *absolute* amounts of food. The curve-fitting analysis that we performed showed that aggression exhibits a continuous increase and then a decrease as the quantity of food is increased from none to intermediate to high amounts. In this chapter, we will attempt to extend this analysis and apply game theory models to explain two main findings: 1) In the presence of females, male flies show a consistent level of aggression, regardless of the number of females, and 2) in the presence of food, male flies increase and decrease the amount of fighting as the amount of food increases from low to an intermediate level to a high level.

Due to the large number of assumptions that we must make in order to apply these models, the contents of this chapter are only included as an Appendix, and the results of these analyses should only be considered preliminary.

Game theoretic models

Game theory models, which are used to model economic behavior in

humans, have been applied to describe the logic of animal conflicts by ethologists and evolutionary biologists (Maynard Smith, 1974; Smith and Price, 1973). These models predict that contests between animals should be conditional, based on assessment of risk (via assessment of opponent's fighting abilities and strategy) and assessment of benefits (via assessment of the resource value). In addition, these models predict that depending on the payoff structures of contests, optimal strategy at the population level can consist of a mixed strategy (that is, there is no single best strategy to always fight or always surrender).

There are two types of decisions that flies can make: 1) They can choose to fight, or compete over the resource ("Fight"), or 2) they can choose to not fight, or give up on taking over the resource ("Peace"). For each pair of actions, we can consider a pair of payoffs by terms a, b, c, d:

a = payoff for each fly when both flies chooses *fight*.

b = payoff when one fly chooses *fight,* and the opponent chooses *peace*.

c = payoff when one fly chooses *peace,* and the other fly chooses to *fight*.

d = payoff when both flies choose *peace*.

		Fly 2	
		Fight	Peace
Fly 1	Fight	a, a	b, c

Peace	c, b	d, d

Game theoretic models for competition over females

Thus far, these are standard conditions of game theory, with no specific assumptions made that pertain to our situation where two male flies are competing over females. In order to rigorously apply game theory to the male-male aggression, certain energetic costs, such as those associated with fighting vs. not fighting, must be measured. Nevertheless, since we do not have data on these measurements, we must make some assumptions based mostly on intuition, not empirical data.

Let us consider the following initial conditions, which are specific to competition over females. We first assume $b > d$; that is, conditional on that the opponent chooses peace, payoff is always higher when the fly chooses to fight. This assumption is natural in that, by choosing peace when the opponent chooses peace, male flies would get nothing, whereas by choosing to fight, male flies would get access to the female. We further assume $a > c$; that is, flies always get more by fighting, regardless of whether the opponent chooses fight or peace. Previous studies support these assumptions, as winning male flies tend to have a higher chance of copulation (Dow and Schilcher, 1975), suggesting that there is a possible payoff for choosing to fight. Although specific energetic considerations are not based on empirical evidence, since *Drosophila melanogaster* males do not

possess weapons and thus cannot easily kill each other, the negative costs associated with choosing to fight may be outweighed by the potential benefits of successfully copulating with the female.

These two assumptions $b > d$ and $a > c$ give rise to a special game called the "prisoner's dilemma game" where both contestants are acting competitively ("anti-coordination"). Its name comes from the situation, where two prisoners face a choice between remaining silent to help the other prisoner vs. betraying each other. Although both prisoners could get the best-case scenario when they both help each other, maximum reward for each player is achieved in the single Nash equilibrium, where each player always acts anti-cooperatively by choosing betrayal. Returning to the game with two male flies, in this prisoner's dilemma game, the pure strategy Nash equilibrium for each fly is to always choose "fight." Regardless of whether the other fly chooses to fight or not, and regardless of the resource availability (number of females), the strictly dominant action (i.e. optimal strategy) would be to "fight," although some experimental variation (i.e. noise) can be expected. These predictions are compatible with the experimental results we observe in Chapter 3 showing that: a) the level of aggression is very high (anti-cooperative), with group-housed flies fighting, and often both male flies participating in the fight, and b) number of females does not seem to affect the level of aggression.

Game theoretic models for competition over food

Now, let us consider the case when flies are competing over food. Once again, we use the same payoff matrix using terms a, b, c, d. Unlike in the case of competing over females, flies that are unstarved should fight in a more conditional manner. Once again, many assumptions that we make here have little to no empirical evidence, because energetic costs associated with each choice are not known. However, unlike competition over females, it should be possible to measure these variables using calories, although such measurements have not been performed. Therefore, once the caloric costs and benefits are measured, the game theoretic model's predictions can be compared with experimental data to test whether fly aggression operates in a strategic manner.

Since most of the fighting occurs on the food surface, the boundary of which is defended (see Chapter 2), let us assume that that flies are choosing to "fight" when they are on the food surface. When both flies are on the food surface, they are both choosing to fight. Next, let us assume that c > a; that is, when both flies engage in aggression, the energetic cost associated is greater than the benefit the fly may obtain by winning the fight. As in the previous case, b > d; that is, if the other fly chooses peace by staying off the food patch, the fly gains more by choosing to fight by staying on the food surface.

The initial assumptions $c > a$ and $b > d$ lead to another special game called the "coordination game." In coordination games, three Nash equilibria (henceforth equilibria) exist. Two equilibria are pure strategy equilibria where one fly chooses to be aggressive and the other fly chooses to be peaceful, vice versa, and one

mixed strategy equilibrium where each fly probabilistically chooses to be aggressive. These equilibria suggest that the optimum strategy in the case of coordination game is a conditional one, compared to the case of the Prisoner's dilemma. Therefore, assuming that the initial assumptions are true, food competition is conditional and more probabilistic compared to mate competition.

Male-male aggression behavioral assays occur over many minutes, and there are many fighting bouts in the assay. Thus, we focus on the third unique mixed strategy equilibrium, as it incorporates randomness of population average that we observe in our data. This unique mixed strategy equilibrium is represented by (π, 1−π) where π is the probability that a fly chooses aggressive action, which implies

$$\pi = \frac{(b-d)}{(c-a)+(b-d)}.$$

How does the coordination game relate to the dose-response curve we observe in the level of aggression with respect to the amount of food? Let's simplify the equation above by letting $b-d=f$ and $c-a=g$. Then, π simplifies to:

$$\pi = \frac{f}{f+g}.$$

g is the motivation for fighting assuming that the opponent does not fight ("motivation for fight"), while f is the motivation for choosing peace assuming opponent fights ("motivation for peace"). Since both terms are positive, π increases

as f increases, while π decreases as g increases.

Although the precise calculations of the probability of fighting, π, require measurements of the terms a, b, c, d , π 's dependency on these terms demonstrates sets numerical constraints, which can be compared to experimentally-observed probability of fighting. For instance, we can measure the energetic costs a, b, c, d in intermale aggression and calculate π, then compare this value with the experimentally-observed level of aggression. This model can thus help us test whether fly competition over food is driven by a strategy compatible with game theory or not.

CONCLUDING REMARKS

In this section, I will briefly summarize all of the findings in the preceding chapters and explore possible future directions, which may be useful.

Summary of findings

As I have covered in the Introduction, many species that exhibit interspecific aggression compete over resources. The presence of resources and the presence of a competitor, who limits the access to such resources, leads to aggression. This leads us to the hypothesis that there may be specific opponent cues and resource cues, as well as neural circuits processing these cues, that control aggression.

Opponent detection mechanisms vary from species to species. In general, they rely on some combination of visual, auditory and chemical pheromone cues, which may function redundantly or dominantly to control aggression. Resource detection mechanisms also vary depending on the resource and species. In general, female resources are detected using similar mechanisms to male opponent detection, while food cues are detected using chemosensory mechanisms.

Much is known about male-specific pheromones, and neural circuits processing these cues as they relate to aggression, but little is known about how resource-specific chemicals or chemosensory mechanisms processing these cues. Thus, I set out to identify resource-specific cues, which control aggression in food and females. In most animals, it is known that food and females increase male-male aggression. However, they are usually not studied independently. I found that

females and food independently increase aggression.

Two common confounds in studying how resources control aggression are that resources tend to be attractive, which may nonspecifically increase encounters between males, and that resources may increase not only aggression, but nonspecifically increase all other behaviors as well. These are important because if the resource's effect to increase aggression is secondary to either increased proximity between males or nonspecific increase in all behaviors, it would imply that animals do not necessarily compete over resources *per se*, but rather that resources indirectly increase aggression. Using the machine-vision-assisted analytical tools, I tested a) whether resource cues were merely increasing encounters, and b) whether resource cues increased aggression specifically or all behaviors by measuring parameters, which were difficult to measure previously, such as proximity, locomotion, and courtship. I found that food and females could increase aggression independently of their effects on encounter duration. Furthermore, I found that food specifically increased aggression and not male-male courtship, while with females, I found that males increased courtship as well as aggression. In both cases, I observed that resources increased locomotion, but the increase in aggression was disproportionate to the amount of increase in locomotion, suggesting that the effect of resources on aggression was not entirely dependent on increases in locomotion.

I went on to characterize the sensory mechanisms by which resources control aggression. With food, I found that sucrose is sufficient to increase

aggression, while for females, I found that female cuticular hydrocarbon pheromones and 7,11-HD are sufficient to increase aggression. Since *Drosophila melanogaster* is a powerful genetic model organism, I also sought to identify chemosensory mechanisms processing these resource-specific cues. For food, I found that the gustatory receptor neurons detecting sugars such as sucrose, *Gr5a*+ GRNs, mediate resource detection in aggression. For females, I found that the gustatory receptor neurons detecting female pheromones—*ppk23*$^{+}$ and *ppk25*$^{+}$ GRNs—mediate resource detection in aggression. Although more experiments are necessary to elucidate the role of *ppk23*$^{+}$ and *ppk25*$^{+}$ GRNs on aggression, these data strongly suggest that male and pheromone detection play a critical role in aggression.

Chemosensory code for male-male aggression in *Drosophila*

By using target male flies and resource female flies, which lack pheromones, I found that there is a dual requirement for both male and female pheromones for male-male aggression. In the presence of one, only changes in male-male or male-female courtship behaviors are observed, but in the presence of both, there is increased male-male aggression. Male and female pheromones are detected by *ppk23*+ and *ppk25*+ GRNs, but they are overlapping populations. To test the binary chemosensory code model for male-male aggression, it is necessary to be able to selectively manipulate male pheromone-sensing GRNs and female pheromone-sensing GRNs.

Thus, I generated genetic tools to separate the function of each population. The newly generated tools, *ppk25-LexA* and *ppk25-GAL80*, function as predicted, as they are expressed in *ppk25*$^{+}$ GRNs. More studies, particularly Calcium-imaging and behavioral experiments, are necessary to test whether *ppk25*$^{+}$ GRNs selectively mark female pheromone-sensing GRNs and whether *ppk23-GAL4*/*ppk25-GAL80* flies can be used to selectively control male pheromone-sensing GRNs. At minimum, these new tools can be used to test whether the proposed model for the roles of *ppk23*+ and *ppk25*+ GRNs in male and pheromone detection is correct. Assuming they are, it should be possible to use these tools to identify possible neural circuit mechanisms of integrating these two pheromonal cues. Previous studies in other laboratories and ongoing studies in our laboratory have identified various *fruitless*$^{+}$ neurons in the brain, which control aggression, such as in the subesophageal ganglion (SOG) (Andrews et al., 2014) and in the lateral protocerebrum (Asahina et al., 2014). By imaging the activity in these regions, it should be possible to test whether the central nervous system responds to either male pheromones or female pheromones or both.

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